Extraintestinal Spread and Replication of a Homologous EC Rotavirus Strain and a Heterologous Rhesus Rotavirus in BALB/c Mice

M. Fenaux, M. A. Cuadras, N. Feng, M. Jaimes, and H. B. Greenberg*

Stanford University School of Medicine, Stanford, California, and Veterans Affairs Palo Alto Health Care System, Palo Alto, California

Received 20 December 2005/Accepted 7 March 2006

Although rotavirus infection has generally been felt to be restricted to the gastrointestinal tract, over the last two decades there have been sporadic reports of children with acute or fatal cases of rotavirus gastroenteritis testing positive for rotavirus antigen and/or nucleic acid in various extraintestinal locations such as serum, liver, kidney, bladder, testes, nasal secretions, cerebrospinal fluid, and the central nervous system. Recently, studies in animals and people have demonstrated that rotavirus antigenemia is a common event during natural infection. In this study, we extend these observations and compare the intestinal and extraintestinal spread of wild-type homologous murine rotavirus EC and a heterologous strain, rhesus rotavirus (RRV), in newborn mice. A strand-specific quantitative reverse transcription-PCR (ssQRT-PCR) assay was used to quantify the ability of different rotavirus strains to spread and replicate extraintestinally. Both strain EC and RRV were detected extraintestinally in the mesenteric lymph nodes (MLN), livers, lungs, blood, and kidneys. Extraintestinal replication, as measured by ssQRT-PCR, was most prominent in the MLN and occurred to a lesser degree in the livers, kidneys, and lungs. In the MLN, strain EC and RRV had similar ($P < 0.05$) RNA copy numbers, although EC was present at a 10,000-fold excess over RRV in the small intestine. Rotavirus nonstructural protein 4 (NSP4) and/or assembled triple-layered particles, indicated by immunostaining with the VP7 conformation-dependent monoclonal antibody 159, were detected in the MLN, lungs, and livers of EC- and RRV-inoculated mice, confirming the ssQRT-PCR findings. Infectious RRV was detected in the MLN in quantities exceeding the amount present in the small intestines or blood. The cells in the MLN that supported rotavirus replication included dendritic cells and potentially B cells and macrophages. These data indicate that extraintestinal spread and replication occurs commonly during homologous and some heterologous rotaviral infections; that the substantial host range restrictions for rhesus rotavirus, a heterologous strain present in the intestine, are not necessarily apparent at systemic sites; that the level and location of extraintestinal replication varies between strains; that replication can occur in several leukocytes subsets; and that extraintestinal replication is likely a part of the normal pathogenic sequence of homologous rotavirus infection.

Rotavirus-associated gastroenteritis affects an estimated 111 million children worldwide and is responsible for at least 440,000 deaths annually (25). Although in some reports rotavirus antigen and/or RNA was detected in the central nervous systems, lungs, kidneys, spleens, heart, testes, bladders, and pancreases of selected severely ill children (19, 20), homologous rotavirus replication has generally been considered to be restricted to the terminally differentiated epithelial cells of the small intestinal villi. The sporadically reported findings of extraintestinal virus have usually been considered to be rare cases, likely due to complicating circumstances in immunocompromised children. Interestingly, extraintestinal spread of homologous mouse rotavirus (EDIM) was first reported in 1958 (17), but since EDIM was found in vascular tissues, it was generally assumed that the virus was simply present in the blood and not actively replicating extraintestinally.

Recently, a study by Blutt et al. demonstrated that the extraintestinal spread of rotavirus, as measured by antigenemia, was a frequent event in otherwise-healthy mice, rats, calves, and humans (2). That study used a qualitative solid-phase

immunoassay to detect viral antigenemia, so neither the question of whether rotavirus was actually replicating in nonintestinal sites nor the question of how much virus could be found systemically versus in the gut were directly addressed. Simian rhesus rotavirus (RRV) was detected sequentially in the Peyer's patches (PP), mesenteric lymph nodes (MLN), livers, spleens, lungs, and blood of 5-day-old CD-1 mice after oral feeding, suggesting that this heterologous rotavirus could use a lymphatic route to escape the small intestine (22, 23, 29). This spread was not demonstrated, however, with a homologous murine rotavirus (30), and it was generally felt that the ability to spread systemically was a relatively unique characteristic of the heterologous RRV strain. Studies of reassortants between RRV (simian rotavirus), which spreads efficiently to the liver, and SA-11 (simian rotavirus), which does not, revealed that gene segment 7 (NSP3) played a dominant role in regulating the spread to the liver and segment 6 (VP6) played a dominant role in regulating escape from the small intestine to the MLN. Of note, both RRV and SA-11 rotavirus are heterologous viruses (simian rotaviruses in a murine host), and the relative efficiency of heterologous versus homologous virus to replicate in the intestine versus spread to or replicate in other organs was not studied (22, 23). Early reports have also shown that RRV, but not a murine rotavirus, was capable of causing hepatitis in immunodeficient SCID mice (30).

* Corresponding author. Mailing address: VA Palo Alto Health Care System, 3801 Miranda Ave., MC154C, Palo Alto, CA 94304. Phone: (650) 752-9722. Fax: (650) 852-3259. E-mail: harry.greenberg@stanford.edu.

The demonstrations of the extraintestinal spread of rotavirus in mice have generally been detected by infectivity titration or enzyme-linked immunosorbent assay (ELISA) of tissue homogenates. Although these are standard and reliable methods, in a recent study when mice were orally administered a plant virus, cowpea mosaic virus, viral RNA and viral antigens were detected in the blood and perfused peripheral organs, including the livers, lungs, kidneys, and brains, from 1 to 3 days postinoculation (28). Since plant viruses are unable to replicate in an animal host, the simple detection of rotavirus infectivity or protein in peripheral organs does not necessarily indicate viral replication. Therefore, our approach was to study rotavirus spread and replication by using several complementary methods capable of detecting rotaviral RNA, antigen, and the presence of several stages of the replication cycle, starting with the transcription of rotaviral (+) sense single-stranded RNA (ssRNA), followed by translation of viral protein and the assembly of rotaviral triple-layered particles and ending with the actual detection of infectious virus.

Murine rotavirus antigens have been detected within PP and MLN in macrophages, B cells, and dendritic cells (DC) but not in other locations (4, 7). However, it was unclear from these studies whether the intracellular rotavirus antigens seen were the result of active replication or passive acquisition. In virtually all immunohistologic studies to date, systemic rotaviral antigens have been visualized with hyperimmune anti-virion antisera, which did not permit the differentiation between replication and simple antigen uptake. However, as proposed by the Brown et al., the relatively large amount of rotavirus antigen detected in the MLN might indicate that rotaviral replication was supported by leukocytes (4).

Recent findings (2, 6, 11, 18, 20, 22, 32) make it clear that rotaviral antigenemia is common in humans and animals and can occur during both homologous and heterologous rotavirus infections. What remains to be seen is whether this spread is associated with extraintestinal replication and how the level of systemic viremia compares quantitatively to the levels seen during intestinal replication.

MATERIALS AND METHODS

Viruses. A stock of wild-type non-cell-culture-adapted murine rotavirus (EC rotavirus) was prepared as an intestinal homogenate, and the titer (the 50% infectious dose [ID_{50}]) was determined by infecting suckling mice as previously described (5). Tissue culture-adapted RRV was prepared as previously described, and the titer was determined by plaque assay (13).

Mice and viral infection. BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred in the Palo Alto Veterans Administration breeding facility. The breeding colony was monitored to ensure it was free of rotavirus contamination. Five-day-old suckling mice were inoculated by gastric gavage with 10^4 ID_{50} of EC RV or 10^7 PFU of RRV (2×10^3 ID_{50}) per mouse. Mice were sacrificed at the indicated time points after infection. Two additional litters of BALB/c suckling mice were inoculated with somewhat higher doses of murine rotavirus (10^5 and 10^6 ID_{50} [EC]) for the collection of MLN for cytospin studies and to determine whether rotaviral viremia is associated with serum or peripheral blood mononuclear cells (PBMC). The use of increased inoculum titers in these additional groups was intended to assure all littermates were infected since samples were pooled. All studies were approved by the Institutional Animal Care Committee.

Sample collection. Samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 15 days postinfection (dpi). At each time point four or more pups from the EC (10^4 ID_{50}) and RRV (10^7 PFU) groups were sacrificed for study. Uninfected BALB/c pups from selected time points were necropsied and used as negative controls. After termination, the mouse skin was treated with ethanol, and any feces or bedding was removed. Mice were not perfused prior to collection of tissue

samples. Tissues were collected in strict order: blood, lung, liver, kidney, MLN, small intestine, and feces to avoid cross-contamination. Blood was collected by heart puncture. An aliquot of whole blood was immediately frozen in liquid nitrogen for strand-specific quantitative reverse transcription-PCR (ssQRT-PCR), and the remainder was left at 4°C for collection of the serum for antigen detection. The thoracic cavity was opened first for the collection of lung tissue, and subsequently the abdominal cavity was opened. The contents of the small intestinal segment were removed if solids were present; however, the small intestines were not flushed in order to better retain the epithelial layer. Instruments were cleaned with ethanol between tissue collections. All samples collected for ssQRT-PCR were immediately snap-frozen in liquid nitrogen, after which the samples were kept at -80°C until use.

ssQRT-PCR assay. Primers F.NSP3.ECRRV (5'-TGCTCAAGATGGAGTC-3') and R.NSP3.ECRRV (5'-GTTTTGACAGTGTAGC-3') were used for the RT of cDNA and amplification of a 1,022-bp fragment from the EC and RRV NSP3 gene. The RT-PCR was performed according to the manufacturer's protocol for QuantiTect SYBR green RT-PCR (QIAGEN, Inc., Valencia, CA). The cDNA fragments of RRV and EC were cloned into the TOPO cloning plasmid (Invitrogen, Inc., Carlsbad, CA). The cloned fragments were partially sequenced (GenBank numbers DQ391186 and DQ391187), and the sequences were used to identify optimum areas on the NSP3 gene to design the EC.C(+) (5'-GTTC GTTGTGCCTCATTCG-3') and EC.C(-) (5'-TCGGAACGTACTTCTGG AC-3') ssQRT-PCR primer pair (422 to 521 bp) and a RRV.C(+) (5'-CCCCG TGTTCAGTGACTC-3') and RRV.C(-) (5'-CGTGGTGAAGTTGAAGTT G-3') ssQRT-PCR primer set (444 to 561 bp). The cloned EC and RRV NSP3 cDNAs were used for the transcription of RNA using mMessage mMachine T7 according to the manufacturer's protocols (Ambion, Inc., Austin, TX). The expressed NSP3 EC and RRV RNAs were separated by gel electrophoresis in 1% Ultrapur Agarose (Invitrogen). The correct bands were excised and washed by using RNaid Spin kit (Q.BIOgene, Irvine, CA), and the RNA concentration was determined by spectrophotometry. The transcribed RNA was diluted and used in the ssQRT-PCR assay with the EC.C(+)-EC.C(-) and RRV.C(+)-RRV.C(-) primer pairs to determine the ssQRT-PCR primer sensitivity. Standard curves during ssQRT-PCR sample assays were determined by using serial dilutions of either the RRV or EC cloned NSP3 cDNA because of plasmid DNA resistance to degradation over time. However, RT, followed by PCR amplification starting from an RNA template, is less efficient than direct PCR amplification from a plasmid-bound cDNA template. Therefore, serial dilutions of RRV and EC NSP3 *in vitro*-transcribed RNA were assayed alongside the respective NSP3 cloned cDNA by serial dilution. The difference in the resulting RNA and DNA standard curves was then used to calculate the RT-PCR efficiency normalization factor relative to the PCR. In addition, the inhibition of PCR due to the presence of PCR inhibitory factors in RNA extracted from the feces, serum, blood, liver, small intestine, lung, and kidney was quantified by running RRV and EC QRT-PCR assays with either EC or RRV virion RNA in the presence of total RNA extracts from respective tissue samples collected from rotavirus-free mice of equivalent age. The levels of inhibition were used to determine tissue- and primer-specific correction factors.

The method to detect and quantify rotavirus genomic double-stranded RNA (dsRNA) versus excess rotavirus plus-sense ssRNA, or strand-specific QRT-PCR (ssQRT-PCR), was based on the following rationale. The rotavirus genome is made up of dsRNA containing one copy of NSP3 plus-sense RNA [(+)RNA] and one copy of NSP3 minus-sense RNA [(-)RNA] hybridized in dsRNA form. When rotavirus infects cells, multiple excess NSP3 plus-sense mRNA copies are expressed for protein translation and the transcription of minus-sense RNA used in progeny genomes. Hence, during replication excess NSP3 (+)RNA strands are present at higher copy numbers than complementary NSP3 (-)RNA. The ssQRT-PCR assay was designed to detect this difference by performing two RT reactions, one specific for the (+)RNA and one specific for the (-)RNA strand of the NSP3 gene. One reaction uses the total RNA extract as a template with only the EC.C(+) primer, complementary to the NSP3 (+)RNA sequence. The opposing reaction, which uses only the EC.C(-) primer, complementary to the NSP3 (-)RNA strand, was used to detect NSP3 (-)RNA. For the reverse transcriptase step, the reactions were incubated at 42°C for 10 min, the reverse transcriptase enzyme was then degraded by heating the sample to 95°C for 15 min. Since the ssQRT-PCRs use a one-step RT-PCR mix, once the RT reaction was finished, the equivalent amount of the opposite primer was added to the reaction, and the Q-PCR step was initiated. For the Q-PCR step, the reactions were transferred to a 384-well ABI optical plate format. The Q-PCR included 36 cycles at 95°C for 10 s, 48°C for 10 s, and 72°C for 25 s. For reactions with RRV samples, the RRV.C(+) and RRV.C(-) primers were used according to a protocol identical to that used for the EC reactions. Groups of PCR results from specific organs at specific time points were evaluated by two sample *t* tests.

Significance was established if P was <0.05 . The data are shown as individual points with a geometric mean.

Statistical determination of rotavirus replication by ssQRT-PCR. We identified a (+)RNA/(−)RNA ratio of >1.58 as indicative of the presence of significant excess (+)RNA over (−)RNA in a tissue sample and hence the presence of rotavirus transcription. This value was selected after the determination of 62 repetitive ssQRT-PCR (+)RNA/(−)RNA ratios on separate rotaviral RNA extracts from EC- or RRV-infected mouse feces. Rotavirus-containing feces was chosen for estimating the normal distribution of the (+)RNA/(−)RNA ratio in a nonreplicating environment, since virus does not replicate in feces and feces contains various nucleases that would be expected to eliminate most or all free ssRNA. Hence, these 62 samples were used to derive an estimate of the natural fluctuation in (+)RNA/(−)RNA ratios that can be expected from *in vivo*-derived samples in which viral replication was not present, further limiting the detection of false positives. The 99th percentile of the normal distribution with a mean of 0.84 and a standard deviation of 0.32 is at a (+)RNA/(−)RNA ratio of 1.58. Therefore, only 1% of samples with no replicating virus would be expected to have a (+)RNA/(−)RNA ratio above 1.58, and any sample with a (+)RNA/(−)RNA ratio greater than 1.58 was defined, for the purpose of this study, as having excess (+)RNA indicative of transcription and hence rotaviral replication.

In addition, we determined that the ssQRT-PCR (−)RNA primers of EC and RRV had a 3- to 4-log reduction in sensitivity when used with the (+) sense RNA NSP3 sequence as a template for ssQRT-PCR. This reduction in the sensitivity of the (−)RNA primers for the opposing template, (+)RNA, makes false priming unlikely, reducing the possibility of overestimation of viral replication. After the last cycle of each reaction, a denaturation cycle was included for the removal of false amplification products. Since differences in assay sensitivity might occur from day to day, an additional EC or RRV rotavirus genomic dsRNA standard was included in each ssQRT-PCR, which allowed for the normalization of the (+)RNA versus (−)RNA primer reaction in each reaction. To limit the presence of (+)ssRNA from these genomic dsRNA isolates, virus isolates from the small intestine (EC) and cell culture (RRV) were first homogenized and then frozen-thawed for the release of virus. The samples were then treated with RNase T₁ (0.01 U/ μ l at 37°C for 15 min) prior to RNA extraction, degrading ssRNA and leaving the genomic virion-associated dsRNA intact.

In vitro test of the ssQRT-PCR during a one-step replication curve. MA104 cell cultures in 24-well plates were inoculated with trypsin-activated RRV at a multiplicity of infection of 1 for 1 h at 37°C, and an additional 24-well plate was left uninfected as a negative control. The cells were subsequently washed five times with phosphate-buffered saline (PBS) and were fed with M199 media with antibiotic but without fetal calf serum and trypsin. Immediately after adding the media, the first time point (1 h) was collected by removing the media and collecting the cells using a cell scraper. The collected cells were immediately frozen in liquid nitrogen. Triplicate RRV samples and one negative control sample were collected every hour until 12 h postinoculation. A fifth RRV-inoculated set of MA104 cell cultures was used to collect at time points 1, 4, 8, and 12 h; these samples were used to determine the RRV titer by plaque assay (13). The MA104 samples were prepared for RNA extraction as described below.

RNA extraction for ssQRT-PCR. All samples were weighed prior to thawing in preparation for the RNA extraction. Total RNA was extracted by using RNAwiz (Ambion, Inc., Austin, TX) according to the manufacturer's protocol. The samples were homogenized with a handheld homogenizer using disposable tips (Fisher Scientific, Pittsburgh, PA). Total RNA was resuspended in 100 μ l of water. The resuspended RNA was heated to 95 to 98°C for 5 min and then immediately frozen in liquid nitrogen. The RNA extract was stored at −80°C until use. Since the samples, such as feces and liver, have various quantities of total RNA, we denoted our results as RNA copies per microgram of sample.

Isolation of peripheral blood leukocytes. In order to determine whether rotavirus RNA detected in the blood was associated with the plasma or leukocytes, an additional litter of 5-day-old mice was inoculated with 10^6 ID₅₀ of EC by gavage. This litter was inoculated with a higher dose to enhance viremia, since preliminary studies detected limited viremia in mice inoculated with 10^4 ID₅₀ EC. At 3 dpi the mice were sacrificed, and blood samples were collected by heart puncture in a syringe containing 20 μ l of sodium citrate. The blood was gently centrifuged at 2,000 rpm to pellet the cells. The plasma was removed and frozen in liquid nitrogen. The isolation of PBMC was performed by diluting the cell pellet in 1 mM EGTA in PBS, adding an equal volume of 2% Dextran T500, and incubating the mixture at 37°C for 20 min. The supernatant was then removed and centrifuged. Red blood cells were lysed by using red blood cell lysing buffer (Sigma-Aldrich, Inc., St. Louis, MO). The cells were diluted in PBS and washed twice. The pelleted PBMC were snap-frozen in liquid nitrogen. Total RNA was extracted from plasma and the collected PBMC as described above. The ex-

tracted rotaviral RNA was then quantified by using the ssQRT-PCR as described.

Detection of RV antigen by IFA. Tissues from 10^4 ID₅₀ of EC-infected mice, 10^7 PFU of RRV-infected mice, or noninfected control mice were collected at selected time points, fixed in 10% buffered formalin, dehydrated, embedded in paraffin, and serially sectioned. The immunofluorescence assay (IFA) conditions were optimized by using a biotinylated mouse monoclonal antibody (MAb) B4-2 to rotavirus nonstructural protein 4 (NSP4) (27). To confirm the presence of rotavirus replication in peripheral tissues, we examined tissue sections for the presence of triple-layered, fully assembled particles by IFA with biotinylated MAb 159 (8, 9, 12), which is specific for an epitope of VP7 found on rotaviral triple-layer particles. The presence of triple-layered particles was interpreted as signifying complete assembly of the infectious rotavirus particle. The cell nucleus was stained with TOTO-3 (Molecular Probes, Eugene, OR).

Detection of RRV by plaque assay. MLN, small intestine, and serum samples were collected at 5 dpi from three mice inoculated by oral gavage with 10^7 PFU of RRV at 5 days of age. The collected samples were prepared by homogenizing the tissues in a 10% (wt/vol) suspension in M199 medium without serum. Rotavirus in the samples was activated by incubation with 10 μ g of trypsin for 20 min at 37°C. Samples were then serially diluted and added to a MA104 monolayer in six-well plates for 1 h. After removal of the inoculum, the monolayers were washed with M199 medium, and the cells were covered by M199 medium with 0.5 μ g of trypsin/ml, $1\times$ antibiotics, and 0.5% agarose. After 3 to 4 days of incubation the agarose was covered by neutral red stain, and the plaques were read (13). Since the murine rotavirus strain EC used in these studies is not cell culture adapted, plaque assay titrations were not performed on EC samples.

Cytospin assay of leukocytes isolated from the MLN. To determine which cells in the MLN supported rotavirus replication, an additional study was performed. A litter of 5-day-old BALB/c mice were inoculated with a 10^5 ID₅₀ of EC. A higher inoculum titer was used in this experiment to enhance the likelihood of homogenous infection of the MLN among individual mice, since the collected cells were pooled. At 4 days after inoculation the animals were sacrificed, and MLN samples were collected and pooled. Leukocytes in the MLN were isolated by mechanical disruption through a wire mesh. Cells were washed in PBS and counted. A total of 10^5 cells were centrifuged onto a microscope slide at $163\times g$ using the Cyto-Tek centrifuge (Miles Scientific, Elkhart, IN). Cells were fixed on the slide with cold acetone and allowed to dry for 1 h. All slides were pretreated in 10% goat serum in PBS. The slides were stained with B4-2 MAb to NSP4 and a secondary goat anti-mouse Texas Red antibody (Southern Biotechnology Associates, Birmingham, AL). The conditions were optimized individually for staining with commercial biotin-tagged marker antibodies to B220 (BD Pharmingen, San Diego, CA), CD11c (eBioscience, San Diego, CA), and CD11b (BD Pharmingen). Background signals were blocked with an Avidin/Biotin Blocking Kit (Vector Laboratories, Inc., Burlingame, CA). The signal for the cell marker antibodies was amplified by using the TSA Fluorescein System (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer's protocol.

Detection of RV antigen by ELISA. The preparation of samples (serum, kidney, liver, lung, MLN, small intestine, and feces) for the detection of RV antigen was as follows. The samples were weighed, and a 10% (wt/vol) suspension in PBS was prepared, except for serum samples, which were tested undiluted. Samples were selected to represent all time points and were otherwise selected randomly. The samples were homogenized with a handheld homogenizer using disposable tips (Fisher Scientific). The homogenized samples were centrifuged at 1,000 rpm for 10 min to pellet cellular debris. Then, 200 μ l of supernatant was collected and incubated with 50 mM EDTA for 10 min at room temperature. The rotavirus antigen sandwich ELISA was carried out as described previously (27). Respective tissue samples collected from noninfected mice were processed as mentioned above as negative controls to determine the background signal. In addition, selected RV antigen positive tissues were assayed by preimmune serum capture as a negative control for nonspecific binding.

Relative sensitivities between ssQRT-PCR and rotavirus ELISA. To enable the comparison between the relative sensitivity of the semiquantitative ELISA and the ssQRT-PCR, two-fold serial dilutions of 10^8 PFU of RRV were analyzed in parallel by ELISA and the ssQRT-PCR. The ssQRT-PCR assay sensitivity limit was previously determined by using a known number RNA copies as a template. ELISA and ssQRT-PCR values were converted into RRV PFU equivalents for the purpose of reporting relative quantities.

RESULTS

Detection and quantification of RV replication in vitro with ssQRT-PCR. A one-step 12-h growth course of RRV in

FIG. 1. A 12-h rotavirus replication curve in MA104 cells inoculated with RRV at an MOI of 1. Bars: □, total (+)RNA copy numbers per ml; ▨, total (-)RNA copy numbers detected per ml. Asterisks mark the time points when a significant excess of total (+)RNA over (-)RNA was detected [(+)RNA/(-)RNA ratio of >1.58], indicating rotavirus replication. The triangles connected with the solid line indicate the RRV titer (in PFU/ml) determined by plaque assay. The x axis shows the hours postinoculation, and the y axis indicates the $\log_{10}$ of both (+)RNA and (-)RNA copies/ml and PFU/ml.

MA104 was analyzed to initially characterize strand-specific detection by the ssQRT-PCR assay. Samples collected from 1 to 4 h postinoculation showed virtually no increase in (-)RNA copy numbers, representative of genomic RNA (Fig. 1). However, from 2 to 10 h postinoculation a significant excess (+)RNA over (-)RNA ((+)RNA/(-)RNA > 1.58) was detected, a finding indicative of rotaviral replication (Fig. 1 and see Materials and Methods). At 5 h postinoculation, an increase in (-)RNA copy numbers over previous time points was first detected. By 11 h postinoculation, there was no longer a significant excess (>1.58) of (+)RNA over (-)RNA. In this ssQRT-PCR assay, increasing titers of genomic (-)RNA at late time points of infection made it difficult to detect a statistically significant excess (+)RNA signal, although it is certainly likely that (+)RNA synthesis was still occurring at these times. The detection of increasing (-)RNA copy numbers, indicative of genomic dsRNA, correlates well with the increasing RRV PFU titer determined by plaque assay (Fig. 1).

Detection and quantification of genomic rotavirus (-)RNA and excess rotavirus (+)RNA in the intestine and feces. (i) Feces. Murine EC rotavirus (-)RNA (genomic RNA) was shed in the feces in ~100-fold-higher copy numbers ($P < 0.05$), from 3 to 8 dpi, than heterologous RRV (Fig. 2 and Table 1). Rotaviral (-)RNA fecal shedding was detected in both EC- and RRV-inoculated mice starting at 1 dpi. RRV was shed until 12 dpi, whereas EC virus was still present in feces on 15 dpi when the study was terminated.

(ii) Small intestine. Over the 15 days of infection, mice inoculated with EC had significantly higher (>10,000-fold) amounts of (-)RNA in the small intestine than RRV-infected

mice (Fig. 2 and Table 1). Except on 1 dpi, the ssQRT-PCR assay did not detect excess EC (+)RNA; this was presumably due to very high levels of (-)RNA in the small intestines of EC-inoculated mice. Nevertheless, the high and sustained level of (-)RNA in the EC-infected mice, as well as IFA microscopic examination of the small intestine demonstrating NSP4 staining (data not shown), clearly supports the presence of active EC replication in intestinal epithelial villus cells. Despite the substantially lower levels of (-)RNA in RRV-infected mice, excess (+)RNA was detected from day 1 through day 12, a finding indicative of low-level RRV replication (Fig. 2). Of note and distinct from EC-infected mice, the levels of RRV (-)RNA in the small intestines were ~10,000-fold lower than in the feces (Fig. 2 and Table 1).

Detection of rotavirus and rotavirus replication in extraintestinal tissues. (i) Blood. Both EC- and RRV-inoculated mice became viremic, as indicated by the detection of (-)RNA starting at 1 and 2 dpi, respectively (Fig. 3 and Table 1). The EC (-)RNA copy numbers in the blood were very low compared to the levels detected in the feces, small intestines, or MLN (Fig. 3 versus Fig. 2 and Table 1), and the same was true for RRV except for the fact that RNA levels in the blood and small intestine were relatively similar. In general, the levels of viremia were similar between EC- and RRV-infected mice despite the substantial differences seen in the small intestine, although on day 5 there was a significantly higher level of (-)RNA in EC-infected mice (Fig. 3). Evidence of rotavirus replication [excess (+)RNA] was not detected in the blood of either EC- or RRV-infected mice.

(ii) **MLN.** The MLN was the extraintestinal site with the greatest amounts of both (–)RNA and excess (+)RNA in both EC- and RRV-inoculated mice (Fig. 3 and Table 1). Rotaviral (–)RNA in the MLN was first detected beginning at 2 dpi and was still present at 15 dpi. In general, the levels of RRV and EC (–)RNA in the MLN were comparable, with significant differences only seen on day 9 (Fig. 3). Although the level of (–)RNA in the MLN of EC-infected mice was ~10-fold lower than the level in the small intestine, the level of (–)RNA in the MLN of RRV-infected mice was actually higher (~100-fold or more) than that seen in the small intestine. For both EC- and RRV-infected mice (–)RNA levels in the MLN clearly exceed those present in the blood, further suggesting the replication of virus at this site.

(iii) **Liver.** Rotaviral (–)RNA was detected in the liver samples of both EC- and RRV-infected mice (Fig. 3). EC (–)RNA was detected in the livers of 32 of 50 mice, and the geometric mean level exceeded that found in the blood by at least 10-fold, suggesting viral amplification in this organ (Table 1). (–)RNA from RRV-infected mice did not exceed the levels in the blood, was significantly lower than EC-infected mice and was only detected in 5 of 50 animals. Replication in the liver, indicated by excess (+)RNA, was detected in 7 EC- and 4 RRV-inoculated mice out of a total of 50 animals (Fig. 3).

(iv) **Lung.** EC and RRV (–)RNA was detected in the lungs of 14/50 and 17/50 of mice, respectively, and the amount of (–)RNA, although higher in RRV-infected mice, was not significantly different from that in EC-infected mice (Fig. 3). EC (–)RNA levels were comparable to those in the blood, and RRV (–)RNA levels were 10-fold higher than in the blood, suggesting viral amplification in this organ (Table 1). The replication of EC, as indicated by excess (+)RNA, was rarely detected in the lungs, and RRV replication was not detected by ssQRT-PCR (Fig. 3).

(v) **Kidney.** Low to moderate copy numbers of EC (–)RNA were detectable from 2 to 8 dpi (Fig. 3). These levels exceeded those present in the blood by ~10-fold (Table 1). RRV (–)RNA was only detected in the kidneys of one animal at 2 dpi. Low levels of excess EC (+)RNA were detected in the kidneys of the majority of inoculated mice between 2 and 8 dpi. Excess RRV (+)RNA was not detected in the kidney.

Rotavirus (–)RNA copy numbers in the bloodstream versus peripheral organs. The (–)RNA copy numbers in tissue samples were individually quantified, enabling the direct comparison between rotaviral (–)RNA in the bloodstream and in peripheral organs of individual animals. The detection of higher (–)RNA copy numbers in the blood than in the MLN of individual animals occurred in only 2/40 EC-infected animals and in none (0/32) of the RRV-infected animals. The blood (–)RNA levels were higher than the liver levels in only 3/32 EC- and 0/5 RRV-infected mice, in the lungs of 6/14 EC- and 0/17 RRV-infected animals, and in the kidneys in 4/18 EC- and 0/1 RRV-infected mice. This comparison supports the conclusion that in the majority of peripheral organs containing detectable EC or RRV (–)RNA, the (–)RNA did not originate from blood contamination. The IFA results discussed below using MAb to rotavirus NSP4 (MAb B4-2) and the VP7 epitope specific for triple-layered particles (MAb 159) further support the conclusion that the excess viral RNA detected extraintestinally was, in part, derived from active extraintesti-

nal replication and did not solely originate from organ trapping of virus originating from the circulation (31).

Rotavirus genomic dsRNA is primarily associated with the plasma fraction of the blood. Using the ssQRT-PCR assay, we determined that EC (–)RNA in the blood is primarily associated with the plasma fraction (Fig. 4). However, a small amount of the EC (–)RNA, but no excess (+)RNA, was associated with the leukocyte fraction.

Rotavirus antigen detection by ELISA in various tissues. Previous studies have primarily demonstrated rotavirus antigenemia by ELISA. In order to compare the current results with previous findings, we tested selected specimens for antigen content by ELISA (Table 1). The ELISA was intended primarily to complement the ssQRT-PCR; therefore, a smaller number of samples from each tissue were tested. Strand-specific QRT-PCR is more sensitive than ELISA. The simultaneous quantitation of tissue culture-derived RRV in our ELISA and ssQRT-PCR assay revealed that the ELISA had a detection limit of 7,500 PFU/ml or 1,500,000 (–)RNA copies/ml of cell culture material. The ssQRT-PCR has a detection limit of 5 PFU/ml or 1,000 RNA copies/ml. Therefore, the ELISA was significantly less sensitive than the ssQRT-PCR assay. Of note, the cell culture-derived RRV stock used for this comparative assay better reflects the nature of rotavirus-containing tissue specimens from mice than purified particle from cell culture since it contains both empty and full triple-layered and double-layered viral particles, as well as free rotaviral antigens.

(i) **Feces.** Rotavirus antigen was detected in the fecal samples of 17/18 EC- and 5/19 RRV-infected mice, which in the case of RRV likely reflects the lower sensitivity of ELISA (Table 1). The feces of EC-inoculated animals contained significantly higher antigen levels (>10,000-fold) than RRV-inoculated animals (Table 1 and Fig. 2). The differences in the fecal antigen levels between EC and RRV were greater than the differences between the (–)RNA levels, although in both instances the homologous murine strain was shed in greater amounts (Table 1).

(ii) **Small intestine.** Rotavirus antigen was detected in 15/18 of EC- and 6/11 of RRV-infected mice, which is generally similar to the results obtained with ssQRT-PCR (Table 1). Higher small intestinal levels of rotavirus antigen (~1,000-fold) were detected in EC- compared to RRV-infected mice, a finding similar to what was seen with the ssQRT-PCR assay.

(iii) **MLN.** RV antigen was detected in the MLN of both EC and RRV-infected mice. RV antigen was detected in equivalent amounts in EC- and RRV-infected mice (Table 1), which is consistent with the ssQRT-PCR results. The antigen levels in the MLN of both EC- and RRV-infected mice were generally 10- to 100-fold higher than the levels found in the blood (Table 1), further supporting replication at this site.

(iv) **Blood.** Low levels of rotavirus antigen were detected in 6/26 of EC and 1/14 of RRV-infected mice. The ELISA results are consistent with the ssQRT-PCR data detecting only low levels of (–)RNA in EC and even less in RRV-infected mice. The antigen levels seen in the blood were less than in the feces, small intestines, or MLN of the same animal.

(v) **Liver.** Although 32/50 [158 ± 10 (–)RNA copies/ μ g] of EC-infected mice were positive for rotaviral (–)RNA, only 1/26 (2 ± 10 PFU equivalents/ml) mice were rotavirus antigen

FIG. 2 and 3. (–)RNA and excess (+)RNA copy numbers detected in the feces, small intestines (SI), MLN, blood, livers, lungs, and kidneys in suckling BALB/c mice inoculated with EC or RRV. The black diamond indicates the (–)RNA copy numbers in the inoculum. The solid black dots (●) indicate the total (–)RNA copies/μg of tissue or feces in an individual animal. The black bar indicates the geometric mean of the data set on each day. The asterisks indicate the time points when rotavirus replication [defined as a significant excess of (+)RNA] was detectable. The “†” symbol indicates that the mean (–)RNA copy numbers are significantly ($P < 0.05$) higher compared to the same day and tissue in the other inoculation group (EC versus RRV). Groups were evaluated by a two-sample t test. Significance was established if the P value was < 0.05 . The x axes show the days postinoculation, and the y axes indicate the $\log_{10}$ of the (–)RNA copy numbers per microgram of tissue.

positive (Table 1). Conversely, RRV (–)RNA was detected in only 5/50 [2 ± 6.3 (–)RNA copies/μg] of infected mice, whereas 10/21 (125 ± 251 PFU equivalents/ml) were positive for rotavirus antigen. The antigen levels in the liver of EC-infected mice were lower than in the blood by 10-fold; however, in RRV-infected mice ~ 10 -fold more antigen was present in the liver than in the blood (Table 1).

(vi) **Lung.** No rotavirus antigen was detected by ELISA in the lung samples of 22 EC-infected mice. Moderate amounts of antigen were detected in 2/14 of lungs from RRV-infected mice (Table 1). The percentages of antigen and the relative levels of antigen detected in the lungs are consistent with the (–)RNA copy numbers detected in both RRV- and EC-infected mice (Table 1).

IFA detection of rotavirus NSP4 and triple-layered particle-dependent VP7 epitope in extraintestinal tissues. Rotavirus NSP4 (MAb B4-2) and the VP7 epitope specific to triple-layered particle (MAb 159) immunostaining was detected in selected tissue sections from the MLN, livers, and lungs of both EC- and RRV-infected mice (Fig. 5). Similar immunostaining results were obtained using hyperimmune anti-rotavirus rabbit serum (data not shown). The presence of NSP4 and/or assembled triple-layered particles in tissue sections from the MLN, livers, or lungs is indicative of the presence of both active viral translation and triple-layered particle assembly and further supports the ssQRT-PCR findings documenting the ability of

both homologous EC and heterologous RRV rotavirus to undergo transcription, translation, and assembly in systemically infected tissues. In the MLN, consistent with the ssQRT-PCR and ELISA results, moderate numbers of NSP4 and triple-layer-particle-positive cells were detected (5 to 20% of cells in field) in both EC- and RRV-infected mice (Fig. 5A1, A2, B1, and B2). Within the lungs, only rare NSP4-positive cells were detectable in both RRV- and EC-infected mice (Fig. 5C1 and C2). In the liver, rotavirus triple-layered particle-staining cells were detected in RRV-infected mice in preliminary studies (Fig. 5D1 and D2). Sections from the kidneys were also tested by IFA, and all were negative for rotavirus antigen.

RRV plaque assay for infectious virus in blood, intestines, and MLN. At 5 dpi, the MLN of three RRV-inoculated mice contained 31,000 PFU/mg ($\pm 23,000$ PFU/mg). In the same animals, the small intestine had a titer of 320 PFU/mg (± 302 PFU/mg) of tissue, whereas all blood samples were negative. The RRV plaque titers are consistent with the ssQRT-PCR RNA findings that RRV (+)RNA and (–)RNA copy numbers are higher in the MLN than in the small intestine or blood and support the previous conclusions that viral replication takes place extraintestinally.

Identification of MLN cells staining for NSP4. In order to begin to identify which cell types outside the small intestine supported rotavirus replication, we first studied the MLN that had the highest level of extraintestinal rotaviral (–)RNA in

FIG. 3.

TABLE 1. Quantities of rotavirus (–)RNA and antigen in suckling mice inoculated with EC or RRV

Sample	Inoculum	Rotavirus (–)RNA		Rotavirus antigen		IFA
		% Positive mice (no. positive/no. tested)	Geometric mean ($\pm$ SD) ^a	% Positive mice (no. positive/no. tested)	Geometric mean ($\pm$ SD) ^b	
Feces	EC	98 (49/50)	6.8 ($\pm$ 0.6)	95 (17/18)	6.2 ($\pm$ 0.9)	NA ^c
	RRV	86 (43/50)	4.5 ($\pm$ 1.0)	26 (5/19)	1.3 ($\pm$ 2.3)	NA
Small Int.	EC	98 (49/50)	5.0 ($\pm$ 1.0)	83 (15/18)	4.7 ($\pm$ 1.8)	+
	RRV	16 (8/50)	0.3 ($\pm$ 0.8)	54 (6/11)	1.2 ($\pm$ 2.4)	+
MLN	EC	80 (40/50)	3.9 ($\pm$ 1.7)	35 (6/17)	3.0 ($\pm$ 2.5)	+
	RRV	72 (32/44)	3.0 ($\pm$ 1.9)	80 (4/5)	2.4 ($\pm$ 3.3)	+
Blood	EC	31 (14/44)	1.0 ($\pm$ 1.1)	23 (6/26)	1.6 ($\pm$ 2.2)	NA
	RRV	13 (6/44)	0.4 ($\pm$ 0.8)	7 (1/14)	0.7 ($\pm$ 1.8)	NA
Liver	EC	64 (32/50)	2.2 ($\pm$ 1.0)	4 (1/26)	0.3 ($\pm$ 1.0)	–
	RRV	10 (5/50)	0.3 ($\pm$ 0.8)	47 (10/21)	2.1 ($\pm$ 2.4)	+
Lung	EC	28 (14/50)	0.9 ($\pm$ 0.9)	0 (0/22)	0	+
	RRV	34 (17/50)	1.6 ($\pm$ 2.0)	14 (2/14)	1.7 ($\pm$ 2.7)	+
Kidney	EC	45 (18/40)	1.8 ($\pm$ 1.2)	0 (0/6)	NA	–
	RRV	3 (1/29)	0	0 (0/3)	NA	–

^a The log value of the geometric mean (–)RNA copy numbers/microgram at 3 to 7 dpi.

^b The log value of the geometric mean of PFU/ml equivalent values of the ELISA titer at 3 to 7 dpi.

^c NA, not available.

RV-infected mice. Mechanically disrupted MLN cells were acetone fixed on a microscope slide and assayed with several leukocyte cell markers. Using this technique, an accurate estimate of the percentage of positive staining cells could not be made but, as seen in Fig. 5, infected cells were not rare. Among these cells, NSP4-positive cells were found to contain for either B220 antibody (Fig. 6A), a B-cell marker also found on plasmacytoid DC; anti-CD11c (Fig. 6C), a DC marker; and anti-CD11b (Fig. 6B), a marker expressed on macrophages, natural killer (NK) cells, granulocytes, and activated lymphocytes. No NSP4 staining was observed in anti-CD3 stained cells (Fig. 6D), a marker expressed on T cells.

DISCUSSION

Over the last two decades, there have been occasional reports of extraintestinal rotavirus isolations (1, 4, 7–9, 13, 15–17, 19, 22, 28). However, these were generally case reports involving one or a limited number of severely ill children. The actual extent of extraintestinal spread in otherwise-healthy children with acute rotavirus gastroenteritis was not known. Studies by Kraft as early as 1958 detected rotavirus infectivity in the serum and peripheral organs of infected mice (17). More recently, RRV, a heterologous simian strain, was shown to have the ability to escape the small intestine and travel to the PP, MLN, and livers of immunocompromised and, to a lesser degree, healthy suckling mice. This trait was initially thought to be highly restricted to the RRV strain (22, 23, 30). Of note, in 2003 Blutt et al. demonstrated that rotavirus antigenemia was, in fact, a common event in infant mice, adult mice, rabbits, rats, calves, and young children during acute infection (2). A variety of recent studies in children and primates have confirmed these observations (6, 11, 16, 18, 20, 32). These studies clearly demonstrated that the spread of rotavirus to the blood-

stream occurs frequently, but the quantity of viremia relative to the amount of virus in the gut has not been investigated, nor has it been determined whether extraintestinal replication accompanies the viremia.

Although rotaviral antigen was detected in the sera of both animals and humans, it was initially unknown whether the antigenemia was associated with viral RNA and thus indicative of viremia. Very recent studies have, however, documented the presence of rotavirus RNA in the serum of infants, primates, and swine during acute infection (1, 2, 6, 11, 18, 32). However,

FIG. 4. Whole blood drawn at 3 dpi from four 5-day-old mice inoculated with a 10^6 ID₅₀ of EC rotavirus. Plasma was separated from the leukocytes, RNA was extracted from the plasma and PBMC fractions, and an ssQRT-PCR assay was used to determine the (–)RNA and excess (+)RNA rotavirus copy numbers in the plasma and leukocyte fractions. The results are reported in rotavirus (–)RNA copies/μg of whole blood. Significant excess (+)RNA was not detected.

FIG. 5. Serial sections from MLN and lungs were collected from 10^4 ID₅₀ EC- and 10^7 PFU RRV-infected and noninfected controls. Rotavirus replication was detected with biotinylated MAbs to rotavirus NSP4 (B4-2) (27) and to VP7 on assembled rotavirus triple-layered particles (MAb 159) (12) and developed with a secondary streptavidin-Texas Red antibody. The cellular nucleus was stained blue with TOTO-3. NSP4 stained EC-infected MLN (A1), RRV-infected MLN (A2), and uninfected MLN (A3). 159 stained EC-infected MLN (B1), RRV-infected MLN (B2), and uninfected MLN (B3). NSP4 stained EC-infected lung (C1), RRV-infected lung (C2), and uninfected control lung (C3). MAb 159 stained RRV-infected liver at $\times 40$ (D1) and $\times 100$ (D2) magnifications. (D3) MAb 159 stained uninfected liver.

the quantity of virus found in blood or tissues versus the amount present in the gut has remained unknown, primarily because many wild-type rotaviruses are poorly adapted to cell culture and so cannot be accurately quantified by classic cell culture techniques. It has also been unclear whether the rotavirus found in the blood and peripheral organs represents virus produced in the gut or is the potential progeny of virus replicating in extraintestinal tissues. Because of these unanswered

FIG. 6. Cytospin of mechanically disrupted MLN collected 3 dpi from mice inoculated with a 10^6 ID₅₀ EC when 5 days old. (A1) Merged photograph of NSP4 (Texas Red) (A2) and monoclonal antibody to B220 (FITC) (A3), cell surface markers for B cells and plasmacytoid DC. (B1) Merged photograph of NSP4 (Texas Red) (B2) and monoclonal antibody to CD11b (FITC) (B3), cell surface markers for macrophages, granulocytes, NK cells, and a subset of DC. (C1) Merged photograph of NSP4 (Texas Red) (C2) and monoclonal antibody to CD11c (FITC) (C3), cell surface markers for DC. (D1) Merged photograph of NSP4 (Texas Red) (D2) and monoclonal antibody to CD3 (FITC) (D3), cell surface markers for T cells.

questions, it has been difficult to fully evaluate the role of viremia in rotavirus pathogenesis or immunity.

In the present study we used a well-characterized murine rotavirus infection model (5, 10, 15, 21, 30) to investigate the extent and quantity of extraintestinal spread and replication of

the homologous murine strain EC versus the heterologous rotavirus strain RRV in infant mice. For this purpose, we designed and developed an ssQRT-PCR assay capable of detecting and quantifying genomic [(-)RNA] and replicating [excess (+)RNA] rotavirus RNA separately with a sensitivity

of 1 to 10 RNA copies per microgram of sample. With this assay we were able to determine when rotavirus was present outside the small intestine, where it was present, how much (–)RNA could be found at various sites, whether the amount of (–)RNA found in an organ exceeded the amount in the blood, and whether rotavirus was replicating, as indicated by excess (+)RNA, at peripheral sites. The ssQRT-PCR assay enabled us to directly compare intestinal versus extraintestinal replication of the homologous EC murine rotavirus and the heterologous simian RRV strain. Our findings confirmed other recent studies documenting the ability of rotavirus virions to spread to the bloodstream and substantially extended these observations by quantifying the presence and/or replication of homologous and heterologous strains in the feces, small intestines, MLN, livers, lungs, and kidneys (Fig. 2 and 3). In addition, our initial studies revealed some of the extraintestinal cell types in which rotavirus appears to initiate replication (Fig. 6).

EC rotavirus replication occurs most efficiently in the intestine. The amount of (–)RNA in the small bowel of EC-infected mice was more than 10-fold greater than that seen in the next most prominent site, the MLN, and ~1,000-fold or greater than the amount found in other organs. Also of note, substantial evidence for host range restriction was seen in the small intestine, with EC (–)RNA levels exceeding RRV (–)RNA by more than 10,000-fold. Host range restriction of rotavirus replication in the gut provides the basis for several rotavirus vaccine candidates.

Surprisingly, rotavirus was found to replicate quite efficiently in the MLN in both EC- and RRV-infected mice. Clearly, the substantial host range restrictive elements present in the small intestine of mice, limiting RRV replication at that site, are not operative in the MLN. RRV (–)RNA (genomic) and excess (+)RNA (replication) in the MLN was equivalent to, or higher than, RRV RNA levels in the small intestine (Table 1 and Fig. 2 and 3). On the other hand, EC (–)RNA was detected in higher numbers (~10-fold) in the small intestine than in the MLN (Fig. 2 and 3 and Table 1). At 15 dpi RRV (–)RNA and/or excess (+)RNA were undetectable in the small intestine and feces. In the MLN, however, RRV replication and rotavirus genomic (–)RNA were still present (Fig. 3). Although the study was terminated at 15 dpi, the data indicate that RRV persists in the MLN longer than in the small intestine. This previously unappreciated and prolonged systemic phase of infection could help explain observations in the mouse that showed intestinal immunoglobulin A titers peaking 1 month after infection and then steadily declining, whereas immunoglobulin G titers continuously increased over time (14, 21). In the EC-inoculated mice rotaviral replication, determined by excess (+)RNA, was not detectable in the MLN at 15 dpi; however, EC (–)RNA was still present in four of four animals tested. It seems likely that EC replication continued to occur in the MLN on 15 dpi but was not detectable due to high (–)RNA copy numbers (Fig. 3). This conclusion is supported by the fact that NSP4 was detected by IFA in MLN sections collected at 15 dpi from EC-infected mice (data not shown). Future studies will be needed to assess the length of persistence of rotavirus RNA in the MLN of mice infected with either homologous or heterologous viruses. Potentially, the MLN might play a critical role in RV escape from the small intestine by providing a site for substantial and prolonged

secondary viral replication. This conclusion agrees with and extends work by Mossel et al., which suggests that RRV spread from the small intestine passes through the MLN before extending to peripheral tissues (22).

Exactly how rotavirus might use the MLN as a portal of entry into the host is not currently known. Previous studies using polyclonal antisera demonstrated that macrophages, B cells, and DC from murine rotavirus-infected mice contained considerable amounts of rotavirus antigen (4, 7). However, in these studies it was not determined whether cells in the MLN supported virus replication or simply internalized rotaviral antigens taken up from the small intestine. Using the ssQRT-PCR assay we demonstrated that, in fact, rotavirus replicates efficiently within the MLN. We then confirmed, using an MAb specific for rotavirus NSP4, that rotavirus nonstructural proteins can be readily visualized in cells in the MLN. Further analysis demonstrated the presence of fully assembled triple-layered particles in selected tissues, as indicated by immunostaining with a triple-layer-specific antibody to VP7 in the MLN and liver (Fig. 5D). Finally, plaque assays of the MLN from RRV-infected mice showed that infectious rotavirus was found at higher levels in the MLN than in the small intestine or blood, confirming our ssQRT-PCR and IFA findings.

Cell surface marker staining was used to demonstrate that DC support rotaviral replication, confirming and extending the recent findings by Narvaez et al. (24). Potentially, B cells and macrophages (Fig. 6) also support rotaviral replication, whereas T cells appeared to be resistant. Since B220, the surface marker used for identification of B cells, is also found on plasmacytoid DC and the macrophage marker, CD11b, also binds NK cells, granulocytes, and selected DC subsets, we are unable to fully confirm that B cells and macrophages are also permissive targets. Future flow cytometry studies involving costaining experiments with multiple cell lineage markers will be needed to explore this issue in detail. However, the ability of rotavirus to infect certain lymphocyte subsets could indicate a potential route of viral spread from the gut to other organs, thus supporting the postulated role of the MLN as the portal of systemic entry. As previously suggested, from the MLN, rotavirus could use leukocytes trafficking as a transport vehicle to either spread through the lymphatic network or the blood (4, 20). Further studies will be required to determine whether the replication of RV in organs such as the liver, kidney, and lungs occurs in cells of lymphoid or epithelial origin.

S. E. Blutt et al. (unpublished data) have detected higher levels of rotavirus antigen in the sera of homologous EC-inoculated mice than the antigen levels we reported here. Despite this apparent discrepancy, the antigenemia in both studies was associated with a surprisingly low level of rotavirus (–)RNA in the blood (Table 1). This highlights the possibility that during rotavirus infection viral antigens not associated with genomic RNA might be released into the bloodstream by some unknown mechanism. This possibility is also supported by the very limited infectivity found in serum samples containing high levels of rotavirus antigen (Blutt et al., unpublished). In the present study we detected an apparent discrepancy between the levels of rotavirus antigen and (–)RNA in the serum. EC (–)RNA levels in serum peaked at approximately 2×10^5 RNA copies/ml (Fig. 3). Although the RV ELISA required the equivalent of approximately 1.5×10^6 rotaviral

RNA copies/ml to produce a positive signal, 23% (6/26) of EC serum samples were positive for RV antigen (Table 1). The molecular basis for the apparent discrepancy between serum antigen and (–)RNA levels is currently unknown. Of interest, when analyzing RRV liver samples, we noted an even greater discrepancy between the level of RV antigen and the level of (–)RNA (Table 1). Less RV antigen was detected in the liver samples of EC-infected mice than in RRV-infected mice, although the EC (–)RNA copy numbers were significantly higher (Table 1 and Fig. 3). Mossel and Ramig (22, 23) detected RRV virus by plaque assay in 26% of RRV-infected mice at 3 and 5 dpi, whereas we detected RRV (–)RNA in the livers of 14% of mice on the same days, supporting the ssQRT-PCR findings. Why RRV-infected mice have more frequently detectable and higher levels of antigen in their livers is not clear, but it is interesting to speculate that this excess antigen might be linked in some manner to the much higher incidence of rotavirus-associated hepatitis seen in RRV-infected mice (30).

Our study detected no evidence for EC or RRV replication in blood, suggesting that blood lymphocytes do not support significant rotavirus replication. In agreement with this conclusion, we showed that in the blood of mice inoculated with EC virus, 99.7% of the viral (–)RNA was associated with the plasma fraction (Fig. 4). These data are consistent with the suggestion that the bloodstream is a mode of transport for rotavirus but not a significant source of replication (2). The failure to detect viral replication in PBMC was not due to the inability of leukocytes to support infection since replication was present in a variety of leukocyte subsets in the MLN (Fig. 6). It is possible that infected lymphocytes from the MLN do not enter the bloodstream or that these lymphocytes are simply present in the blood at concentrations below the detection sensitivity of our assay.

In the small intestine of EC-infected mice excess (+)RNA was not detectable, presumably because of the concurrent high level of (–)RNA in the feces and small intestine (Fig. 2). In the presence of high numbers of (–)RNA, the ssQRT-PCR assay was unable to accurately detect a statistically significant excess of (+)RNA over (–)RNA (Fig. 1). However, significant EC replication in the small intestine was demonstrated by the high and sustained numbers of (–)RNA in the intestine (Fig. 5) and by immunohistochemical studies (data not shown) (3). Of interest, the level of EC (–)RNA in the intestine was substantially higher (~10,000-fold) than the level of RRV (–)RNA and appeared to be sustained longer (Fig. 2). This finding confirms studies of fecal shedding and immunohistologic studies of the small intestine, demonstrating a clear and substantial growth advantage of the homologous EC strain over even the relatively mouse adapted heterologous simian RRV strain (5). An interesting observation is that there appears to be a relative lack of correlation between the amount of RRV (–)RNA found in the feces and that found in the small intestine. The (–)RNA levels in the feces seem inappropriately high given the (–)RNA levels in small intestine. This lack of correlation appears to extend to the relationship between antigen levels and (–)RNA levels in the feces of RRV-infected mice as well (Table 1). The explanation for these observations is not yet clear, but one possible explanation would be that in RRV-infected mice, significant amounts of virion (–)RNA make their way into the gut lumen via the biliary tract rather than

directly from infected intestinal epithelial cells. This hypothesis is currently being tested.

It seems clear from this and other studies that homologous murine rotaviruses are capable of replicating much more efficiently in intestinal epithelial cells than heterologous rotaviruses. At present both the genetic and the molecular basis for this host range advantage is unknown, but it forms the basis for several human rotavirus vaccine candidates. It is also clear that EC viral replication is far more efficient in the gut than in the extraintestinal sites. Even in the MLN of EC-infected mice, where considerable replication occurs, the amount of (–)RNA was ~10-fold lower than in the gut (Table 1 and Fig. 2 and 3). Strikingly, however, the high degree of host range restriction observed in the small intestine (a >10,000-fold excess of EC versus RRV) was not maintained in the MLN, where replication appeared to be virtually identical between the two virus strains. The reason for this difference is also unclear, but certainly the MLN does not restrict RRV replication or does not permit enhanced EC replication in the same manner as intestinal epithelial cells. One of the hypotheses concerning the association of the RRV-derived Rotashield vaccine with intussusception is that rare instances of MLN hyperplasia in vaccine recipients might have been responsible. From these studies we can conclude that RRV replicates as efficiently as a homologous murine strain in the MLN of mice. Whether this is true for all heterologous rotavirus strains in the mouse model and whether high levels of MLN replication are a common finding in other species, including humans, remains to be determined.

EC (–)RNA was detected at significantly higher levels than RRV (–)RNA in the liver, but this difference was of substantially less magnitude than that seen in the gut (Fig. 3). This observation was surprising since RRV infection, but not EC infection, has been associated with liver disease in mice (22, 23, 26, 29, 30). This may indicate that the heterologous RRV is generally less able to spread to the liver relative to homologous rotavirus strains. On the other hand, substantially more RRV antigenic material was detected in the liver, a finding consistent with its increased hepatotoxicity. It is currently unclear why RRV appears to be considerably more hepatotoxic than EC, but apparently this is not due simply to increased levels of RRV replication in the livers of infected mice. Excess (+)RNA copy numbers, which are indicative of rotaviral replication in the liver, were detected in EC-infected mice and sporadically in RRV-infected mice. In addition, an epitope on VP7 found specifically on assembled triple-layered particles was detected within liver cells by immunofluorescence microscopy. EC replication was further confirmed in hepatic tissues, since EC (–)RNA copy numbers in the liver were higher (10-fold) than in the blood (Table 1 and Fig. 3). As for RRV-infected mice, the few mice with (–)RNA-positive livers did not have (–)RNA in the blood (see Results). In addition, given the substantial replication of RRV seen in the liver of immunodeficient mice (30), we think it is likely that both RRV and EC replicate at low levels in the BALB/c mouse liver. In our study neither the biliary tract or the gallbladder were directly examined to see whether either EC or RRV have increased affinity for these tissues. This will be done in future studies.

Although the presence of rotavirus in the respiratory system has been previously reported (1, 22), rotavirus replication in the lungs had not been demonstrated previously. EC rotavirus

replication was detected in the lungs by ssQRT-PCR and confirmed in both EC- and RRV-infected animals by IFA using antiserum to NSP4 (Fig. 5B1 and B2). The amount was small, especially compared to the gut or even the MLN. In addition, in 8 of 14 EC-infected mice the (–)RNA copy numbers in the lungs were higher than the comparable copy numbers in the blood (Table 1 and Fig. 3), suggesting that EC replication in the lungs occurs in approximately half of the animals studied. In the RRV (–)RNA-containing lungs, the (–)RNA copy numbers were 100- to 10,000-fold higher than in the blood, indicating that the virus detected in infected lungs probably did not originate from the circulation. However, we only detect limited replication by IFA and failed to detect any excess (+)RNA by ssQRT-PCR, suggesting the possibility that the RRV (–)RNA detected in the lungs might have originated in the upper respiratory tract and passively been shed into the lungs. It is interesting that Azevedo et al. (1) have described an attenuated human rotavirus that may preferentially replicate in the upper respiratory tract and nasal passages of pigs (see also reference 22).

Rotavirus RNA and antigen have been detected in the kidneys and bladders of children (20), and the urinary tract might represent an alternative route of viral spread. The ability of EC to replicate within the kidney and the inability of RRV to do the same is the most significant difference between EC and RRV infection outside of the small intestine (Fig. 2 and 3). It is interesting that both the intestine and the kidney are organs with large numbers of highly polarized epithelial cells, and perhaps it is in these cells that one sees the greatest degree of rotavirus host range restriction.

Overall, the extraintestinal spread and replication of EC is slightly more efficient than RRV, especially considering the EC virus titers and replication in the liver and kidney. However, the striking differences between EC and RRV replication in the gut are generally absent in most systemic organs and especially in the MLN. Both the heterologous RRV and the homologous EC viruses have the ability to escape the small intestine, spread systemically, and replicate in peripheral organs. For EC, extraintestinal replication is at levels substantially less than in the small bowel, but this is not necessarily so in RRV-infected animals. Hence, our findings clearly demonstrate and support previous findings (23) that extraintestinal escape is not necessarily directly dependent on how well a virus replicates in the small intestine. We conclude that rotaviral extraintestinal spread and replication is not limited to heterologous or homologous viruses and is part of the normal pathogenic process of rotavirus infection. Multiple approaches have been used to document extraintestinal replication, and these include the presence of excess (+)RNA (transcription) in various tissues, the presence of excess (–)RNA over the that found in blood in various tissues, the presence of cells containing NSP4 and an epitope of VP7 unique to the assembled triple-layered particle, and finally the presence of excess infectious RRV, determined by plaque assay, in the MLN compared to the small bowel and the blood of infected mice. The role of extraintestinal replication in the pathogenesis of the disease and in the host immune responses requires further investigation.

ACKNOWLEDGMENTS

We especially thank Alex Macmillan for assistance with the statistical analysis.

This study was supported in part by a VA Merit Award and NIH grants R01 AI21362 and P30DK56339.

REFERENCES

1. Azevedo, M. S., L. Yuan, K. I. Jeong, A. Gonzalez, T. V. Nguyen, S. Pouly, M. Gochner, W. Zhang, A. Azevedo, and L. J. Saif. 2005. Viremia and nasal and rectal shedding of rotavirus in gnotobiotic pigs inoculated with Wa human rotavirus. *J. Virol.* **79**:5428–5436.
2. Blutt, S. E., C. D. Kirkwood, V. Parreno, K. L. Warfield, M. Ciarlet, M. K. Estes, K. Bok, R. F. Bishop, and M. E. Conner. 2003. Rotavirus antigenaemia and viraemia: a common event? *Lancet* **362**:1445–1449.
3. Boshuizen, J. A., J. H. Reimerink, A. M. Korteland-van Male, V. J. van Ham, J. Bouma, G. J. Gerwig, M. P. Koopmans, H. A. Buller, J. Dekker, and A. W. Einerhand. 2005. Homeostasis and function of goblet cells during rotavirus infection in mice. *Virology* **337**:210–221.
4. Brown, J. W., and P. A. Offit. 1998. Rotavirus-specific proteins are detected in murine macrophages in both intestinal and extraintestinal lymphoid tissues. *Microb. Pathog.* **24**:327–331.
5. Burns, J. W., A. A. Krishnaney, P. T. Vo, R. V. Rouse, L. J. Anderson, and H. B. Greenberg. 1995. Analyses of homologous rotavirus infection in the mouse model. *Virology* **207**:143–153.
6. Chiappini, E., C. Azzari, M. Moriondo, L. Galli, and M. de Martino. 2005. Viraemia is a common finding in immunocompetent children with rotavirus infection. *J. Med. Virol.* **76**:265–267.
7. Dharakul, T., M. Riepenhoff-Talty, B. Albini, and P. L. Ogra. 1988. Distribution of rotavirus antigen in intestinal lymphoid tissues: potential role in development of the mucosal immune response to rotavirus. *Clin. Exp. Immunol.* **74**:14–19.
8. Dormitzer, P. R., D. Y. Ho, E. R. Mackow, E. S. Mocarski, and H. B. Greenberg. 1992. Neutralizing epitopes on herpes simplex virus-1-expressed rotavirus VP7 are dependent on coexpression of other rotavirus proteins. *Virology* **187**:18–32.
9. Dunn, S. J., J. W. Burns, T. L. Cross, P. T. Vo, R. L. Ward, M. Bremont, and H. B. Greenberg. 1994. Comparison of VP4 and VP7 of five murine rotavirus strains. *Virology* **203**:250–259.
10. Feng, N., J. W. Burns, L. Bracy, and H. B. Greenberg. 1994. Comparison of mucosal and systemic humoral immune responses and subsequent protection in mice orally inoculated with a homologous or a heterologous rotavirus. *J. Virol.* **68**:7766–7773.
11. Fischer, T. K., D. Ashley, T. Kerin, E. Reynolds-Hedmann, J. Gentsch, M. A. Widdowson, L. Westerman, N. Puh, R. M. Turcios, and R. I. Glass. 2005. Rotavirus antigenemia in patients with acute gastroenteritis. *J. Infect. Dis.* **192**:913–919.
12. Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino. 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. *J. Virol.* **47**:267–275.
13. Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian. 1984. Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization. *J. Infect. Dis.* **149**:694–702.
14. Ishida, S., N. Feng, B. Tang, J. M. Gilbert, and H. B. Greenberg. 1996. Quantification of systemic and local immune responses to individual rotavirus proteins during rotavirus infection in mice. *J. Clin. Microbiol.* **34**:1694–1700.
15. Ishida, S. I., N. Feng, J. M. Gilbert, B. Tang, and H. B. Greenberg. 1997. Immune responses to individual rotavirus proteins following heterologous and homologous rotavirus infection in mice. *J. Infect. Dis.* **175**:1317–1323.
16. Kehle, J., C. Metzger-Boddien, F. Tewald, M. Wald, J. Schuurmann, and G. Enders. 2003. First case of confirmed rotavirus meningoencephalitis in Germany. *Pediatr. Infect. Dis. J.* **22**:468–470.
17. Kraft, L. M. 1958. Observations on the control and natural history of epidemic diarrhea of infant mice (EDIM). *Yale J. Biol. Med.* **31**:121–137.
18. Li, N., and Z. Y. Wang. 2003. Viremia and extraintestinal infections in infants with rotavirus diarrhea. *Di Yi Jun Yi Da Xue Xue Bao* **23**:643–648.
19. Lynch, M., B. Lee, P. Azimi, J. Gentsch, C. Glaser, S. Gilliam, H. G. Chang, R. Ward, and R. I. Glass. 2001. Rotavirus and central nervous system symptoms: cause or contaminant? Case reports and review. *Clin. Infect. Dis.* **33**:932–938.
20. Lynch, M., W. J. Shieh, K. Tatti, J. R. Gentsch, T. Ferebee-Harris, B. Jiang, J. Guarner, J. S. Bresee, M. Greenwald, S. Cullen, H. D. Davies, C. Trevenen, S. R. Zaki, and R. I. Glass. 2003. The pathology of rotavirus-associated deaths, using new molecular diagnostics. *Clin. Infect. Dis.* **37**:1327–1333.
21. McNeal, M. M., and R. L. Ward. 1995. Long-term production of rotavirus antibody and protection against reinfection following a single infection of neonatal mice with murine rotavirus. *Virology* **211**:474–480.

22. Mossel, E. C., and R. F. Ramig. 2003. A lymphatic mechanism of rotavirus extraintestinal spread in the neonatal mouse. *J. Virol.* **77**:12352–12356.
23. Mossel, E. C., and R. F. Ramig. 2002. Rotavirus genome segment 7 (NSP3) is a determinant of extraintestinal spread in the neonatal mouse. *J. Virol.* **76**:6502–6509.
24. Narvaez, C. F., J. Angel, and M. A. Franco. 2005. Interaction of rotavirus with human myeloid dendritic cells. *J. Virol.* **79**:14526–14535.
25. Parashar, U. D., E. G. Hummelman, J. S. Bresee, M. A. Miller, and R. I. Glass. 2003. Global illness and deaths caused by rotavirus disease in children. *Emerg. Infect. Dis.* **9**:565–572.
26. Petersen, C., D. Biermanns, M. Kuske, K. Schakel, L. Meyer-Junghanel, and H. Mildenberger. 1997. New aspects in a murine model for extrahepatic biliary atresia. *J. Pediatr. Surg.* **32**:1190–1195.
27. Petrie, B. L., H. B. Greenberg, D. Y. Graham, and M. K. Estes. 1984. Ultrastructural localization of rotavirus antigens using colloidal gold. *Virus Res.* **1**:133–152.
28. Rae, C. S., I. W. Khor, Q. Wang, G. Destito, M. J. Gonzalez, P. Singh, D. M. Thomas, M. N. Estrada, E. Powell, M. G. Finn, and M. Manchester. 2005. Systemic trafficking of plant virus nanoparticles in mice via the oral route. *Virology* **343**:224–235.
29. Ramig, R. F. 2004. Pathogenesis of intestinal and systemic rotavirus infection. *J. Virol.* **78**:10213–10220.
30. Uhnoo, I., M. Riepenhoff-Talty, T. Dharakul, P. Chegas, J. E. Fisher, H. B. Greenberg, and P. L. Ogra. 1990. Extramucosal spread and development of hepatitis in immunodeficient and normal mice infected with rhesus rotavirus. *J. Virol.* **64**:361–368.
31. Verdin, E. M., S. P. Lynn, B. N. Fields, and E. Maratos-Flier. 1988. Uptake of reovirus serotype 1 by the lungs from the bloodstream is mediated by the viral hemagglutinin. *J. Virol.* **62**:545–551.
32. Zhao, W., M. Xia, T. Bridges-Malveo, M. Cantu, M. M. McNeal, A. H. Choi, R. L. Ward, and K. Sestak. 2005. Evaluation of rotavirus dsRNA load in specimens and body fluids from experimentally infected juvenile macaques by real-time PCR. *Virology* **341**:248–256.