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Active Viremia in Rotavirus-Infected Mice

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Rotavirus circulates extraintestinally in animals used as models for rotavirus infection and in children. Rotavirus infection in mice was used to define host or viral factors that affect rotavirus viremia. Antigenemia was observed with homologous and heterologous rotaviruses, and neither age nor mouse strain genetics altered the occurrence of rotavirus antigenemia or viremia. Rotavirus RNA and infectious virus were present in sera and associated with the plasma fraction of blood in all infected mice. These findings indicate that antigenemia/viremia occurs routinely in rotavirus infections and imply that infectious rotavirus has access to any extraintestinal cell within contact of blood.

Rotavirus infection and disease are worldwide health concerns resulting in 111 million episodes of diarrhea in children <5 years of age (25). Initially, it was thought that rotavirus infection was restricted to the gastrointestinal tract. However, the detection of rotavirus proteins or RNA outside the intestine (7, 9, 16, 18–20, 23) suggested that rotavirus infection is not limited to the intestine. Extraintestinal rotavirus has been attributed to infections with specific rotavirus strains or in children with immunologic defects (14). However, we and others have demonstrated that proteins and RNA of rotavirus can be commonly detected in the sera of children infected with rotavirus (2, 7, 13).

The mouse model has been widely utilized to define the pathogenesis of rotavirus (5, 12, 15, 27, 31). Both homologous and heterologous rotaviruses have been shown to cause viremia in both infant and adult mice (2, 17, 21). Rotavirus has also been associated with two migrating cell populations isolated from lymph nodes of infected mice, B cells and macrophages (4), suggesting that rotavirus viremia can be both plasma and cell associated. However, rotavirus antigenemia was found to be plasma associated rather than cell associated in piglets (1). In the studies described here, we further investigated the properties of rotavirus viremia in mice and demonstrate the predominant association of the virus with the plasma fraction of blood.

Rotavirus antigenemia does not depend on dose of viral inoculum or on genetic strain or age of mouse. Six- to eight-week-old female outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) were inoculated with 10 or 10^5 50% infectious doses (ID_{50}) of the murine rotavirus strain EC_{wt} (G3P[17]) (12), 10^4 ID_{50} of the murine rotavirus strain EDIM (G3P[17]) (31), 10 ID_{50} ($\sim 10^9$ PFU) of the rhesus rotavirus strain RRV (G3P5[3]) (28), or an equivalent amount of inactivated RRV (6). To detect antigenemia, fecal and serum samples collected from individual mice were analyzed by enzyme-linked immunosorbent assay (ELISA) (24). Samples with an optical density at 450 nm of >0.100 were considered positive for virus. Antigenemia was detected concurrently with fecal rotavirus excretion at both low and high ID_{50} inocula (Fig. 1). Antigen was not detected in fecal or serum samples collected prior to 24 h post-viral inoculation. Antigenemia required replication because inactivated RRV did not result in antigenemia (Fig. 1C). This conclusion is consistent with the report that in piglets, nonreplicating virus-like particles do not cause antigenemia (1).

We recently reported that susceptibility to rotavirus infection is genetically determined in mice (3). To determine whether genetic background influenced antigenemia, several mouse strains (CD-1, CF-1, BALB/c, C57BL/6, and 129) were orally inoculated with a dose of EC_{wt} equivalent to 10^5 ID_{50} in CD-1 mice. Antigenemia was present and approximately equivalent in all mouse strains examined (data not shown), indicating that rotavirus antigenemia does not depend on host genetics.

To determine whether rotavirus antigenemia was dependent on age, three litters of five-day-old CD-1 pups (Charles River Laboratories) were orally inoculated with 10^5 ID_{50} EC_{wt}. Four days after inoculations, sera and intestines from each litter were pooled and tested for rotavirus antigenemia. All sera and intestinal homogenate pools from EC_{wt} inoculated mice were antigen positive (Fig. 2A), indicating that rotavirus antigenemia also occurs in infant mice.

Rotavirus antigenemia and viremia are associated with the plasma fraction of blood. Whole blood collected using lithium heparin or potassium EDTA 3 to 4 days after inoculation of CD-1 mice with 10^5 ID_{50} EC_{wt} was separated into plasma and cell fractions. Each fraction was analyzed for rotavirus antigenemia by ELISA or for infectious virus by testing the ability of the sample obtained from the donor mouse to cause rotavirus fecal excretion in a naïve mouse (recipient). Rotavirus antigen was detected in the plasma but not the cell fraction (Fig. 2B). Both sera and plasma, but not cell lysates, collected

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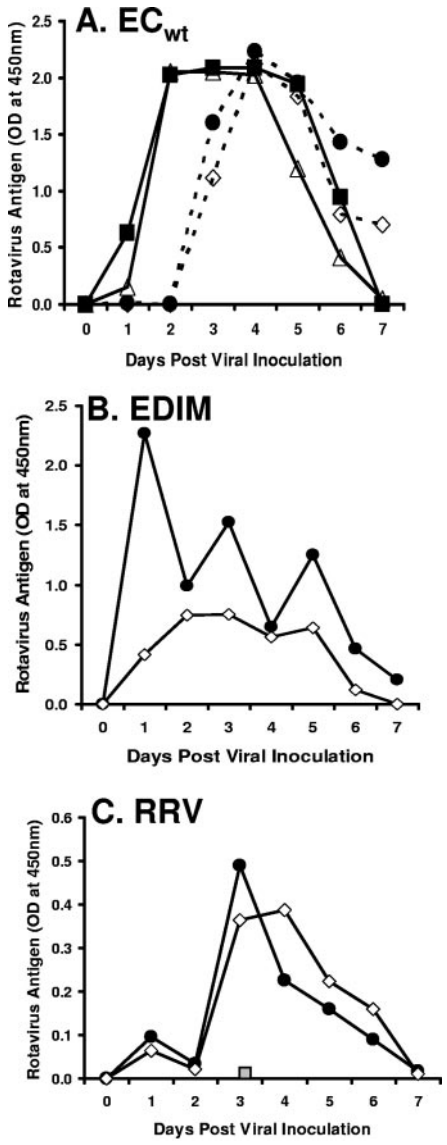

FIG. 1. Rotavirus antigenemia is not rotavirus strain dependent. Rotavirus naïve adult CD-1 mice were orally inoculated on day 0 with the indicated strains of rotavirus. Mice were sacrificed at the indicated time points and 10% (wt/vol) fecal suspensions (squares and circles) and undiluted sera (open diamonds and open triangles) analyzed for rotavirus antigens by ELISA. Points represent average values for each time point ($n = 3$ to 5). A. Mice were inoculated with either 10 ID_{50} (dashed lines) or 10^5 ID_{50} (solid lines) EC_{wt}. B. Mice were inoculated with 10^4 ID_{50} EDIM. C. Mice were inoculated with 10 ID_{50} RRV. The gray box indicates mice inoculated with inactivated RRV ($n = 3$).

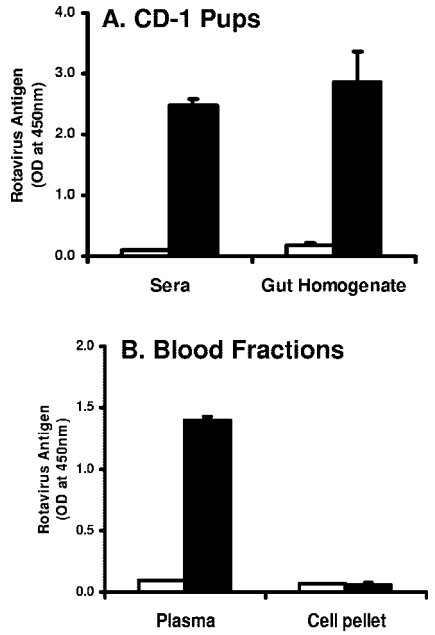

FIG. 2. Detection of rotavirus antigens. A. Six litters of 5-day-old naïve CD-1 pups were randomized (6 to 8 mice/litter) and administered either phosphate-buffered saline (PBS) (white bars, $n = 3$ litters) or 10^5 ID_{50} EC_{wt} (black bars, $n = 3$ litters). Four days after inoculation, the intestines and the blood from each litter were pooled, gut homogenates or sera recovered, respectively, and samples analyzed for rotavirus antigens by ELISA. Bars represent average values of samples pooled from each of three litters ± the standard deviation. B. Naïve adult CD-1 mice were orally inoculated with 10^5 ID_{50} EC_{wt} (black bars) or PBS (white bars). Three days after inoculation, whole blood was collected in EDTA-containing collection tubes for each mouse to prevent clotting. Cells were recovered by low-speed centrifugation after a washing and cell lysates made by addition of 150 μl PBS to each tube followed by three freeze-thaw cycles. Plasma and cell fractions were analyzed by ELISA for the presence of rotavirus antigens. Each bar represents the average value from three mice ± the standard deviation.

from EC_{wt} inoculated infant or adult donor mice resulted in rotavirus fecal shedding in recipient mice (Table 1). Neither plasma collected using EDTA (resulting in the generation of noninfectious double-layered particles) nor sera, plasma, or cell lysates isolated from uninfected donor animals initiated infection in recipient mice (Table 1). Infection of infant mice with homologous rotavirus resulted in antigenemia (Fig. 2A), viremia (Table 1), and disease at 4 days postinoculation (data not shown); but heterologous rotavirus viremia was observed only at 24 to 48 h after inoculation (22), suggesting that the kinetics of homologous and heterologous rotavirus viremia may differ in pups or that viremia is dependent on virulence of the infecting strain. Another explanation may involve the difference in PFU/ID_{50} ratio which has been reported to be 10^4 to 10^5 times higher for RRV than EC_{wt} (12). Our findings indicate that active plasma-associated viremia is a prominent feature in rotavirus infection. Rotavirus has been detected in mouse lymph node macrophages, dendritic cells, and B cells (4, 11), as well as infectious virus isolated from blood cells from immunocompromised mice inoculated with the live Rotashield vaccine (26), suggesting that rotavirus viremia is also cell associated. Our lack of detection of rotavirus in blood cells suggests that cell-associated viremia occurs at low levels or in small numbers of circulating cells. Further work is needed to determine the possible role of cell associated viremia in rotavirus pathogenesis and whether it is a common feature of infections in humans and other animals.

Quantification of infectious virus present in sera from mice excreting rotavirus. Two approaches were utilized to estimate the amount of infectious virus present in sera. First, donor sera from EC_{wt} infected mice were diluted 1:10 and administered to naïve recipient mice. Only one of six naïve recipient mice

TABLE 1. Infectious virus is present in the plasma fraction of sera from EC_{wt}-infected mice[a]

Donor sample	Donor antigenemia	Donor age	Recipient mice infected/total[b]
Serum	−	Adult	0/6
	+	Adult	6/6
Diluted serum[c]	+	Adult	1/6
Serum pool	−	Pup	0/3
	+	Pup	3/3
Plasma	−	Adult	0/3
	+	Adult	6/6
Cells	−	Adult	0/3
	+	Adult	0/6
Plasma/EDTA[d]	+	Adult	0/3

[a] To detect infectious virus, sample was collected at 3 to 4 days postinfection from an EC_{wt}-inoculated mouse (donor) and administered to a naïve mouse (recipient).
[b] Number of naïve recipient mice excreting rotavirus antigen after oral inoculation with a sample from donor mouse/total number of mice inoculated.
[c] Serum samples diluted 1:10.
[d] Plasma obtained after treatment of whole blood with 25 mM EDTA, which prevents clotting by Ca^{2+} chelation and also removes the outer capsid proteins from infectious virus (to yield double-layered particles which are not infectious).

TABLE 2. QRT-PCR determination of total viral RNA levels in sera and fecal suspensions from EC_{wt}-infected mice[a]

Mouse	Value for indicated sample type[b]:			
	Serum samples		Fecal samples	
	RNA[c]	Protein[d]	RNA[c]	Protein[d]
1	1,668	2.316	192,000,000	2.996
2	0	0.236	381,000	2.122
3	1,236	2.433	240,000,000	3.020
4	0	2.781	28,380,000	3.030
5	1,452	2.532	240,000	2.908

[a] Samples were collected 3 days postinfection.
[b] Serum samples were analyzed undiluted, and fecal samples were analyzed as 10% (wt/vol) solutions.
[c] Numbers indicate average copies of double-stranded RNA/μl.
[d] Numbers indicate optical density at 450 nm.

became infected with rotavirus, suggesting the infectious virus titer in the sera is low (Table 1). Second, the number of double-stranded RNA copies/μl was quantitated at the peak of antigenemia by quantitative reverse transcriptase PCR (QRT-PCR), as described previously (11). For QRT-PCR, a primer pair specific to the EC_{wt} NSP3 gene was used. Sera from infected mice contained double-stranded RNA (Table 2), but the amount varied greatly (0 to 1,236 copies/μl). Similar variation in copy number was observed in feces (2.4×10^5 to 2.4×10^8 copies/μl), but the level in feces greatly exceeded that in sera (Table 2). The low RNA copy number was not consistent with the high amounts of protein detectable in both sera and feces by ELISA. The apparent difference in rotavirus RNA and protein levels in sera and feces suggests the production of excess amounts of free viral proteins, release of noninfectious rotavirus particles that do not contain RNA, or disruption of virus and degradation of viral RNA in sera. Fischer et al. (13) reported discrepancies in which human serum samples were rotavirus positive by ELISA and rotavirus negative by RT-PCR and vice versa, supporting the idea that there is a discordance in the results between the two methods in the detection of rotavirus in the blood. Similar discrepancies were also observed between ELISA and QRT-PCR results for rotavirus-infected neonatal mice (11). However, studies in rat pups have shown a correlation between antigenemia and infectious virus (10). Further studies are necessary to determine whether rotavirus protein, RNA, and infectious virus in the blood do not always agree due to methodology issues or biologic differences.

The identification of rotavirus viremia raises important questions as to whether viremia occurs solely as a result of intestinal replication or whether replication of virus at extraintestinal sites also contributes to viremia. Our work demonstrates that rotavirus replication is necessary for viremia to be established in mice, and the kinetics of antigen detection in feces compared to that in sera indicates that antigenemia lags behind intestinal replication. Although it is experimentally difficult to prove unequivocally, the suggestion that intestinal replication precedes the presence of antigen or infectious virus in the blood is supported by kinetic studies with mouse pups (11, 22), rats (10), and pigs (1). The presence of infectious rotavirus within the circulatory system provides one explanation for the findings of rotavirus at extraintestinal locations (30). Determination of whether the virus in the circulatory system represents virus produced in the intestine, at extraintestinal sites, or both in the intestine and at extraintestinal sites will require development of a model in which viremia and intestinal replication are discordant or methods that can differentiate the origin of viral replication, neither of which are currently available.

One additional consequence of viremia, beyond infection of extraintestinal tissues, is the enhancement of intestinal infection. Three previous findings support the idea that rotavirus viremia could result in enhanced intestinal infection: (i) in vitro results demonstrating that Caco-2 cells can be infected by rotavirus at the basolateral surface (8, 29), (ii) intravenous inoculation of gnotobiotic piglets with rotavirus results in intestinal virus shedding (1), and (iii) subcutaneous and intraperitoneal administration of RRV to neonatal mice results in intestinal infection (21). Clearly, infectious virus circulating in the blood may gain retrograde access to the intestine, as well as most tissues. However, the lack of technical approaches to separate the circulatory system from the intestine and extraintestinal organs limits our current ability to address the source of rotavirus viremia and its impact on intestinal and extraintestinal infection. New approaches are needed to gain more information as to the source of rotavirus viremia and its contribution to rotavirus pathogenesis.

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