Kinetics of Viral Replication and Local and Systemic Immune Responses in Experimental Rotavirus Infection

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Rotavirus-seronegative mice were orally inoculated with murine rotavirus in order to study the kinetics of rotavirus replication and the relationship of viral replication to immunity and disease and to assess the effects of local and systemic antibodies on viral clearance and disease resolution.

Rotaviral diarrhea is a significant cause of morbidity and occasionally mortality in many mammalian species including humans, swine, cattle, sheep, horses, and mice (3, 6). Although significant progress has been made in understanding the molecular aspects of rotavirus replication, fundamental questions regarding the natural history and pathogenesis of rotavirus infections remain to be answered. Specifically, although it has been shown that local antibodies provide protection against symptomatic rotavirus infection (1, 8, 9) and that rotavirus infection induces an intestinal antibody response (2, 5, 10, 11), the role of local immunity in viral clearance and disease resolution is not fully understood. Additionally, the kinetics of viral replication and quantitation of rotavirus antigen after experimental infection and their relationship to immunity and disease have not been elucidated.

In this study, we used a murine model of rotavirus diarrhea, epizootic diarrhea of infant mice (EDIM) (4), to examine the kinetics of rotavirus replication and the relationship of viral replication to the immune response and to the development and resolution of disease. Since age has been shown to be a critical factor in EDIM virus infection and disease (7, 12), we examined mice at several ages in order to more fully explore these relationships.

The stock EDIM virus intestinal suspension was produced in suckling Swiss mice. The remainder of the animals used in the study were pregnant, CD-1 mice purchased from a commercial supplier (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Only seronegative animals were used for the studies. Seronegative animals were placed in positive-pressure ventilation isolators and allowed to give birth naturally. Suckling mice remained with their dams throughout the experiment. Immediately before EDIM virus inoculation, mice were transferred from the isolators, and preinoculation serum samples were collected. The study included five experimental groups of suckling mice which were orally inoculated with 10^6 50% mouse infectious doses of EDIM virus at either 1, 7, 14, or 28 days of age. Within these groups, five subgroups, each consisting of eight animals, were terminated on days 1, 3, 5, 7, and 10 postinoculation. At termination, serum and intestinal samples were collected from each animal. The intestinal tracts were evaluated grossly for the presence of diarrhea, carefully dissected to remove mesentery, and rinsed in a physiological saline

solution. Three of the eight intestinal tracts collected from each subgroup were processed for immunofluorescence (7). The intestinal tracts from the remaining five animals in each subgroup were frozen at -70°C for enzyme immunoassays. In preparation for EDIM virus antibody and antigen immunoassays, individual intestinal tracts were thawed, ground in hand-held, Tenbroeck tissue grinders, diluted to 10% (wt/vol) suspensions with phosphate-buffered saline containing 0.01% CaCl_2 , and dissociated further by brief sonication.

Intestinal murine rotavirus antigen was quantitated by using an enzyme immunoassay similar to one described previously (14). In this assay, guinea pig antiserum to EDIM virus was immobilized on the solid phase, followed by the test sample, mouse anti-EDIM virus serum, rabbit anti-mouse immunoglobulin G conjugated to peroxidase, and substrate. The positive control standard on each microtiter plate was arbitrarily assigned a value of 100, and all readings were adjusted accordingly by using the following formula: adjusted test value = $(100/\text{positive control value}) \times \text{test reading}$. This was done to adjust for plate-to-plate variation.

A previously described enzyme immunoassay blocking system (13) was modified and used to detect EDIM virus antibodies in sera and intestinal homogenates. In this technique, the test sample was first incubated with a fixed amount of EDIM virus preparation. The assay was then completed as described above for antigen detection. The antibody activity was determined by measuring the amount of unbound antigen remaining in the sample. Adjusted values for graphic display were determined from each plate by using the following formula: adjusted value = antibody-negative control standard reading/test sample reading. With this formula, values of 1 or less indicated that free antibodies were not present in the test sample to bind to the exogenous EDIM virus and prevent its attachment to the microtiter plate; thus, the test sample gave a reading approximately equivalent to the antibody-negative control standard.

Diarrhea developed only in mice that were inoculated at 1 or 7 days of age (Fig. 1). The levels of intestinal rotavirus antigen were consistently higher at 1 day postinoculation in these mice than in older animals which were resistant to disease. Thus, disease correlated with the quantity of rotavirus antigen detected by enzyme immunoassay. In animals inoculated at 1 or 7 days of age, an initial EDIM virus antigen peak was observed in the intestine 1 day after inoculation; this was followed by a decline at day 3 and a second antigen peak at day 5 postinoculation. The second peak was followed by a rapid decline through days 7 and 10, although base-line antigen levels were not reached. The peak intesti-

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FIG. 1. Intestinal rotavirus antigen (●) and antibodies (○), serum rotavirus antibodies (□), and disease incidence in seronegative, CD-1 mice inoculated with EDIM virus at either 1 (a), 7 (b), 14 (c), or 28 (d) days of age. The antigen assay consisted of guinea pig anti-EDIM virus serum immobilized on the solid phase and mouse anti-EDIM virus serum and enzyme-labeled rabbit anti-mouse immunoglobulin G as detector antibodies.

nal antigen levels at 1 day postinoculation were higher in the animals that were inoculated at 7 days of age than in the animals that were inoculated at 1 day of age. The reverse was true of the second peak observed 2 days later (Fig. 1).

Intestinal samples from mice inoculated at 7 days of age were analyzed for rotavirus antigen by using a second enzyme immunoassay. In this assay goat anti-human rotavirus serum was adsorbed to the solid phase, followed by the test sample, guinea pig anti-EDIM virus serum, peroxidase-conjugated goat anti-guinea pig immunoglobulin G, and substrate. A second antigen peak was not observed with this assay. The antigen levels peaked at 1 day postinoculation and progressively fell thereafter (Fig. 2). One possible explanation for this finding is that murine intestinal rotavirus antibodies may have bound to rotavirus antigens in the form of immune complexes and thus may have increased the signal generated by the interaction with enzyme-labeled anti-mouse immunoglobulins. Alternatively, it is possible that the reagents to mouse rotavirus detected a form of rotavirus antigen that was produced late in infection and was not detectable with reagents raised to human rotaviruses.

Rotavirus antigen was also detected in the intestines of mice inoculated at 14 days of age; however, in contrast to the mice inoculated at either 1 or 7 days of age, the antigen levels in the 14-day-old mice were substantially lower 1 day after inoculation, peaked at a higher level on day 3, and then quickly returned to base-line levels by day 7 postinoculation

FIG. 2. Intestinal rotavirus antigen in seronegative CD-1 mice inoculated with EDIM virus at 7 days of age. The antigen assay consisted of goat antiserum to human rotavirus immobilized on the solid phase and guinea pig anti-EDIM virus serum and enzyme-labeled goat anti-guinea pig immunoglobulin G as detector antibodies.

TABLE 1. Rotaviral diarrhea and distribution of rotavirus antigen in the gastrointestinal tract, as determined by immunofluorescence

Age at inoculation (days)	No. with diarrhea/total no.	Days post-inoculation	No. of animals fluorescent antibody positive/no. of animals tested ^a		
			Proximal intestine	Middle intestine	Distal intestine
7	2/8	1	3/3 (+,+,++)	3/3 (+,+,++)	3/3 (+,+,++)
	8/8	3	3/3 (+,+,+)	3/3 (+,+,+++)	3/3 (+,+,++)
	6/8	5	2/3 (+,+)	3/3 (+,+,++)	3/3 (+,+,++)
	0/8	7	0/3 (0)	0/3 (0)	2/3 (+,+)
	0/9	10	0/3 (0)	0/3 (0)	0/3 (0)
14	0/8	1	3/3 (+,+,+)	3/3 (+,+,++)	3/3 (+,+,++)
	0/8	3	3/3 (+,+,+)	3/3 (+,+,+)	3/3 (+,+,+)
	0/8	5	3/3 (+,+,+)	3/3 (+,+,+)	3/3 (+,+,+)
	0/6	7	0/3 (0)	0/3 (0)	0/3 (0)
	0/6	10	0/3 (0)	0/3 (0)	0/3 (0)

^a Intestines were excised and divided into three areas corresponding to the proximal small intestine, middle small intestine, and distal small intestine. Sections obtained from each area were stained with anti-SA-11 serum, and the proportion of stained cells was expressed as follows (data in parentheses): 0, no visible antigen; +, up to 5% of the cells were fluorescent antibody positive; ++, between 5 and 20% of the cells were fluorescent antibody positive; +++, 20% or more of the cells were fluorescent antibody positive.

(Fig. 1). Only minimal viral replication was detected in mice inoculated at 28 days of age (Fig. 1).

Although the intestinal rotavirus antigen levels in mice inoculated at either 7 or 14 days of age differed and a marked difference in disease incidence was observed (Fig. 1), the extents and distributions of rotavirus antigen as assessed by immunofluorescence were similar in these two groups (Table 1). Thus, the ability of 14-day-old mice to resist disease was not simply related to an inability of the virus to infect certain portions of the intestinal tract. An analysis of antigen quantity by an enzyme immunoassay showed that substantially less expression of viral antigens occurred in the animals inoculated at 14 days of age compared with the animals inoculated at 7 days of age.

Intestinal antibodies directed at rotavirus were first detected in all four groups on day 7 after inoculation (Fig. 1). Significant levels of antibodies were found in all animals after infection, including those in which only limited intestinal viral replication occurred. However, the magnitude of the immune response varied among the groups. Mice inoculated at 1 day of age had the lowest level of free antibodies, which reached its peak 7 days postinoculation, and maintained this level for the remainder of the study (Fig. 1). Mice inoculated at 7 and 14 days of age developed the highest titers of blocking antibodies; these titers reached peak levels at days 10 and 7 postinoculation, respectively (Fig. 1). Animals inoculated at 28 days of age developed intermediate levels of antibody, which were at the highest point on the final day of the study, 10 days postinoculation (Fig. 1). Importantly, this suggests that rotavirus antigens are capable of inducing a local immune response despite low levels of viral replication in the gastrointestinal tract. This finding might have significance in the study of active immunization with rotavirus since it is possible that low levels of viral replication might result in the development of local and systemic immune responses.

Serum antibody titers were determined only in animals inoculated at 1 or 28 days of age (Fig. 1). Antibodies were present at low levels 5 days postinoculation in both groups, and the level continued to rise slightly throughout the 10-day study period. The levels of serum rotavirus antibodies were similar in both age groups.

We were unable to show that the development of antirotaviral intestinal antibodies was sufficient by itself to bring about disease resolution or viral clearance. Thus, in mice

inoculated at 1 day of age, disease and high levels of rotavirus antigen persisted throughout the 10-day study period despite the development of local and systemic antibodies. The increase in intestinal antibody levels which occurred 7 days postinoculation in animals inoculated at 7 days of age coincided with a rapid decline in intestinal EDIM virus antigen and with resolution of disease. This alone might suggest that local antibodies are important in viral clearance and disease resolution. However, at 7 days postinoculation these mice were 14 days old, which corresponds to the time when mice become both naturally resistant to disease and capable of limiting viral replication by mechanisms unrelated to immunoglobulin. The mechanisms of viral persistence in the presence of local antibodies will be the subject of future investigations.

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