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# Age-Dependent Rotavirus–Enterocyte Interactions[1] (41410)

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*Abstract.* The nature of rotavirus–intestinal cellular interaction was examined in duodenal and jejunal enterocytes obtained from groups of adult and suckling mice orally inoculated with murine rotavirus (MRV). The techniques of immunofluorescence (IFA), electron microscopy (EM), and rosetting of MRV-coated sheep erythrocytes (SRBC) were employed for these studies. Immunofluorescence studies demonstrated the presence of viral antigen in the cytoplasm in 2 to 30% of enterocytes isolated from suckling mice but not in any of the enterocytes from adult mice. The peak age of mice with MRV-positive enterocytes was 6–11 days. In all instances enterocytes were isolated 24 hr following oral inoculation of mice with control media or MRV. Isolated dispersed enterocytes from uninfected adult or suckling mice were incubated with purified MRV-coated red blood cells. Specific binding of virus coated SRBC to enterocytes, as evidenced by formation of rosettes, was most pronounced in enterocytes obtained from suckling mice under 11 days of age while only low levels of rotavirus binding activity persisted in the enterocytes from mice older than 75 days of age. These data suggest that the degree of replication of MRV in the intestine may be determined by the availability of virus-specific receptors on enterocytes. The differences in the relative proportion of such receptors between suckling and adult mice may explain the unique predilection of infants to rotavirus infection. Although the pathogenesis of mouse rotavirus closely resembles human rotavirus infection, the identification of similar receptors on human enterocytes remains to be established.

Rotaviruses are ubiquitous in most mammalian species and represent a major cause of acute gastroenteritis in human infants and young children (1–3). Although many enteric viruses gain entry in the human host through the mucosal portal of the gastrointestinal tract, few if any, cause histopathologic or clinical evidence of local intestinal disease. On the other hand, rotaviruses induce pathologic lesions in the intestinal mucosa, alterations on brush border enzymes and functional abnormalities in duodenal mucosa (4–8). The viruses infect the intestinal epithelial cells. Although rotavirus has been recovered from the fecal specimens of subjects in all age groups, clinical disease appears to be uncommon in the neonate and in infants over 30 months of age. The spectrum of clinical illness and the pathogenesis of rotavirus infection in other mammalian species appear to be quite similar to human infection (9–13). Little information is available regarding the mechanisms underlying the age restriction of the clinical disease and the factors which define virus intestinal epithelial cell interaction in human and other mammalian rotavirus infections.

The present studies were undertaken to examine the characteristics and temporal kinetics of rotavirus intestinal epithelial cell interaction in a mouse model employing induced infection with epidemic diarrhea of infant mice (EDIM) agent, a murine rotavirus (MRV) whose pathogenesis resembles that of infection with human rotavirus in man.

**Materials and Methods.** *Animals.* Swiss mice randomly bred ranging in age from 2 days to 6 months were obtained commercially from the West Seneca Breeding Lab-

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oratory, West Seneca, N.Y. All animals were maintained in strict isolation facilities after virus inoculation.

*Virus preparation.* Mouse rotavirus (EDIM 5099) was kindly supplied by Dr. Richard Wyatt, NIH Bethesda, Maryland. The virus pools were prepared from the homogenate of small intestine of 7- to 10-day old suckling mice infected with MRV stock. The MRV-infected intestinal homogenates were clarified by centrifugation at 7000 rpm to remove heavy cellular debris. The virus was pelleted by ultracentrifugation at 35,000 rpm in an SW 50 rotor for 3 hr. The resultant pellet was sonicated (10 sec/ml) in 0.5 ml of phosphate-buffered saline (PBS) and layered onto a 15 to 45% discontinuous sucrose gradient which was further ultracentrifuged at 30,000 rpm for 2 hr. Following this, two distinct bands were visible in the gradient. Fractions (0.1 ml each) were collected. The fractions containing the visible band were dialyzed against normal saline for 24 hr. The final product was monitored for the presence of rotavirus by electron microscopy and enzyme-linked immunosorbent assay (ELISA).

*Inoculation with mouse rotavirus (MRV).* Groups of mice were inoculated with MRV according to the following schedule. Mice 2 to 8 days of age were administered 0.1 ml of the virus intraorally by instillation. Mice 9 to 24 days of age and over 25 days of age were inoculated with 0.1 and 0.2 ml, respectively, of the virus administered via nasogastric instillation. The animals in each age group were sacrificed 24 hr after the inoculation of the virus and intestinal epithelial cells were prepared from segments of proximal small intestines and noninfected age-matched controls inoculated with physiologic saline were included for each infected group and studied in an identical manner.

*Isolation of enterocytes.* Epithelial cells were dissociated from the duodenum and jejunum of uninfected or infected mice using a modification of the procedure described by Weisser (14). Briefly, a 6- to 8-cm segment of the proximal small intestine was removed. The segment was dissected free of fat and connective tissue and cut open longitudinally. The tissue was washed in a balanced buffered saline solution containing 0.2% bovine serum albumin. The washed segment was placed in approximately 10 ml of a buffered saline solution containing 0.5 m*M* ethylenediaminetetraacetic acid (EDTA) (1.86%) and agitated gently for 5 min. The resulting dissociated cells were washed and the segment was subjected to another 5 min of agitation. All cells were pooled and washed twice in standard buffer containing $Ca^{2+}$ and $Mg^{2+}$ salts with no EDTA. The isolated cells, were found to be 90% enterocytes morphologically when examined by light and electron microscopy (Fig. 1) and were employed for further testing as described below.

The enterocytes remained 95% viable (as judged by trypan blue exclusion) for at least one hour following isolation. Twenty-four hours later, less than 30% of the enterocytes were found to be viable.

*Preparation of MRV-coated red cells.* Purified and concentrated MRV (250 $\mu$g/ml of protein) was attached to sheep red blood cells (SRBC) by the techniques of Poston (15) using chromium chloride. Briefly SRBC were suspended in piperazine buffer (pH 6.5) and incubated at room temperature with 0.1 ml of purified MRV in the presence of 2.25 *M* chromium chloride for 4 min. Control RBC were prepared similarly using uninfected small intestinal homogenate in place of MRV. The reaction was stopped by the addition of 3 ml of saline and the cells were washed three times in saline. The coated red cells and similarly treated uncoated red cells were fixed in 1.25% gluteraldehyde and stored at 4° until utilized further.

The presence of virus coating of RBC was determined by membrane immunofluorescence employing fluorescein isothiocyanate (FITC)-conjugated rotavirus antisera produced in gnotobiotic pigs (kindly supplied by E. Bohl and Linda Seif, Veterinary Institute, Wooster, Ohio). Figure 2 demonstrates the specific staining of the MRV-coated RBC (right frame) and the lack of stain on the controls (left frame). The relative proportion of SRBC staining

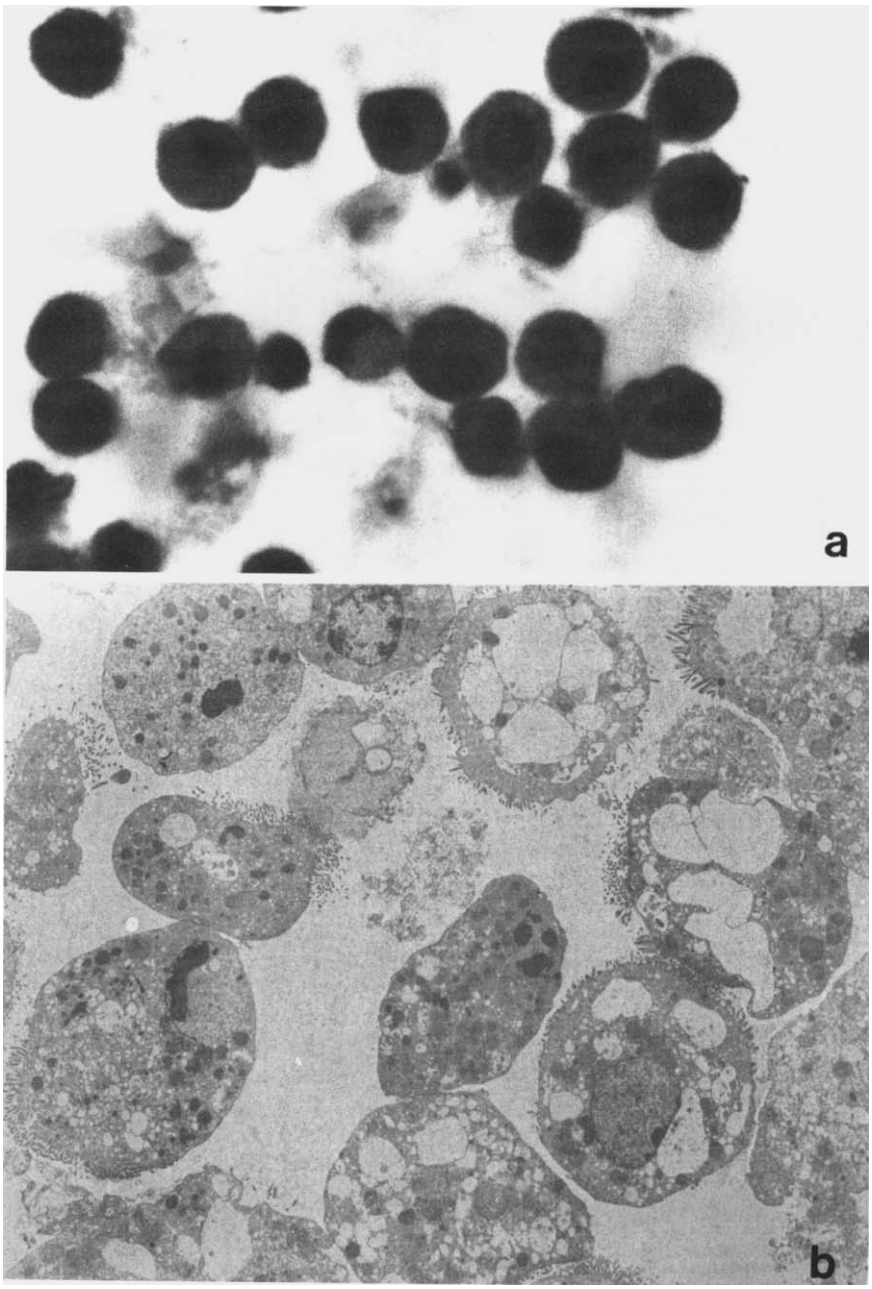


FIG. 1. Micrographs of isolated enterocytes. (a) Light microscopy of hemotoxylin and eosin-stained jejunal enterocytes (400×). (b) Electron microscopy of a thin section of jejunal enterocytes (3000×).

for MRV was approximately 80–90% of total RBC.

*Enterocyte–MRV-coated RBC rosettes.* Washed, isolated intestinal enterocytes obtained from uninfected animals of different age groups were mixed with an equal volume (0.1 ml) of MRV-coated RBC. The mixtures were centrifuged at 700 rpm for 5

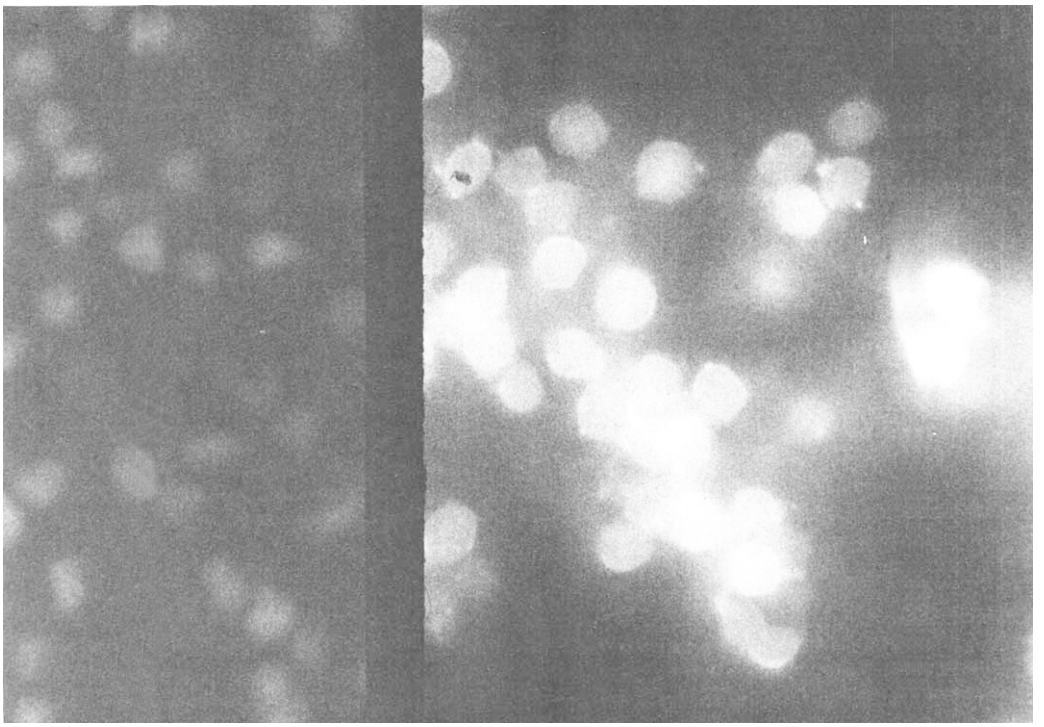

FIG. 2. Membrane immunofluorescence using FITC-labeled porcine antirotavirus of uncoated (left frame) and MRV-coated (right frame) sheep red blood cells.

min and the pellet of enterocytes and MRV-coated RBC was separated and incubated at 4° for 30 min. The preparation was examined on a hemocytometer with a light microscope for the presence of RBC rosettes with enterocytes (Fig. 3). At least 2000 enterocytes were examined in each preparation. Binding of three or more RBC to an enterocyte was considered as an enterocyte–RBC rosette. In addition to the control described above, the specificity of the rosetting for MRV and enterocyte was ascertained by specific blocking procedures. Incubation of enterocytes with purified MRV prior to the addition of MRV-coated RBC effectively blocked rosette formation with such enterocytes.

*Detection of MRV antigen in enterocytes.* The enterocytes obtained from infected mice were tested for the presence of MRV by employing direct immunofluorescence. Air-dried smears of washed dispersed intestinal enterocytes were made on glass slides. This was followed by fixing in acetone at 4° for 10 min. The fixed cells were incubated with a 1:20 dilution of FITC conjugated anti-rotavirus serum for 45 min at 37°. The cells were washed three times in phosphate-buffered saline (PBS). The cells were read on a microscope equipped with a mercury vapor bulb (Leitz, Ortholux, Wetzlar, West Germany). A minimum of 100 cells was examined in each preparation. The number of cells with positive staining for rotavirus antigen was calculated and the percentage of positive cells from each mouse was recorded. Each experiment included appropriate uninfected controls and blocking procedure to rule out nonspecific immunofluorescence.

*Direct electron microscopy.* A small amount of intestinal extract, mixed with 2% ammonium acetate, was dropped onto a carbon-formvar-coated copper mesh grid (Ernest Fullam Co., Schenectady, N.Y.). After removal of excess sample with filter

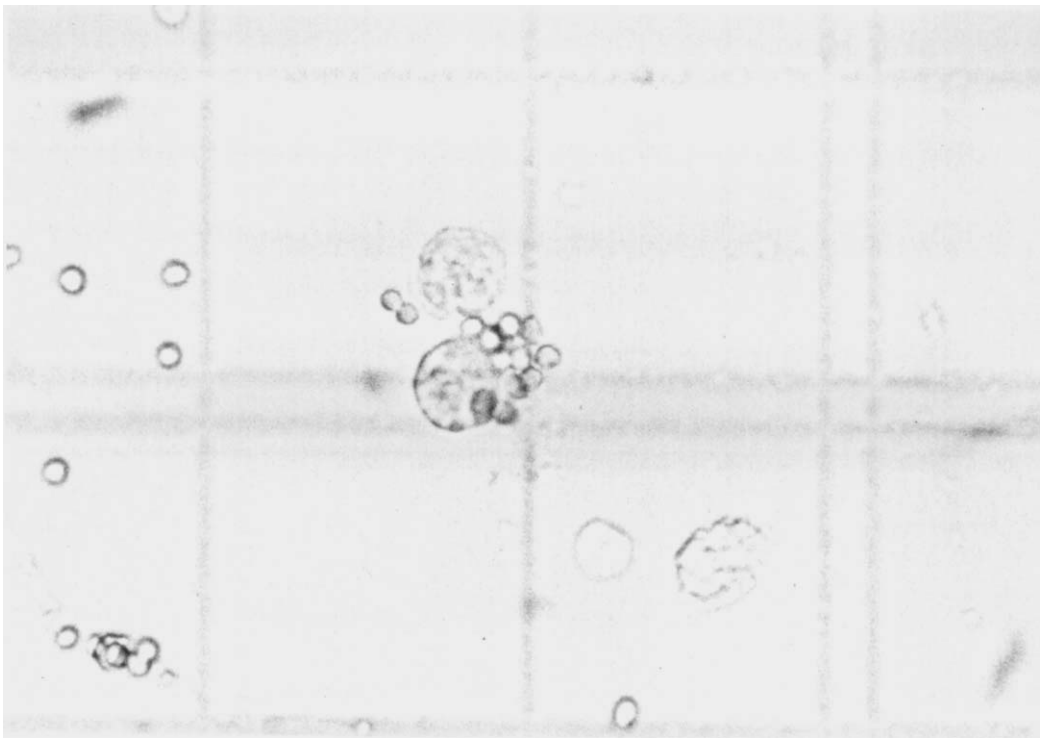

FIG. 3. Micrograph of an enterocyte–SRBC$^{MRV}$ rosette (490×).

paper a drop of 1% phosphotungstate (PTA), pH 7.2, was added to the grid. After approximately one minute, the excess PTA was removed and the grid was placed 2 in. from an ultraviolet light for 3 to 5 min to inactivate viral infectivity. The grids were examined at 30,000× using a JEOL 100S electron microscope (JEOL, Tokyo, Japan).

*ELISA.* The "sandwich" enzyme immunoassay technique adapted from the procedure described by Yolken and colleagues (16) was utilized. The commercially available kit termed Rotazyme was purchased from Abbott Laboratories. Plastic beads coated with guinea pig hyperimmune anti-rotavirus serum, supplied in the kit, were incubated with the sample of intestinal extract. After washing the beads four times with buffer, rabbit anti-rotavirus peroxidase-conjugated serum was incubated with the coated beads. The unbound material was removed by washing and the peroxidase substrate (*O*-phenylenediamine·2HCl) was added. After 15 min of incubation at room temperature the resulting absorbance was read using a spectrophotometer set at 492 nm (Beckman Inst., Fullerton, Calif.).

**Results.** *Characteristics of cell–virus interaction after MRV infection in vivo.* Following oral administration of the virus in the suckling mice, presence of MR virus antigen could be frequently demonstrated in the cytoplasm of isolated enterocytes. A representative pattern of the immunofluorescent staining is presented in Fig. 4. However, the frequency of viral antigen-positive cells appeared to be strictly age dependent. One hundred percent of mice ranging in age from 2 to 11 days (when first infected with MRV) exhibited the presence of virus in the enterocytes. The proportion of enterocytes which contained virus ranged from 12 to 25% of total cells in the age group. The frequency of animals with virus containing enterocytes was lower in those mice in-

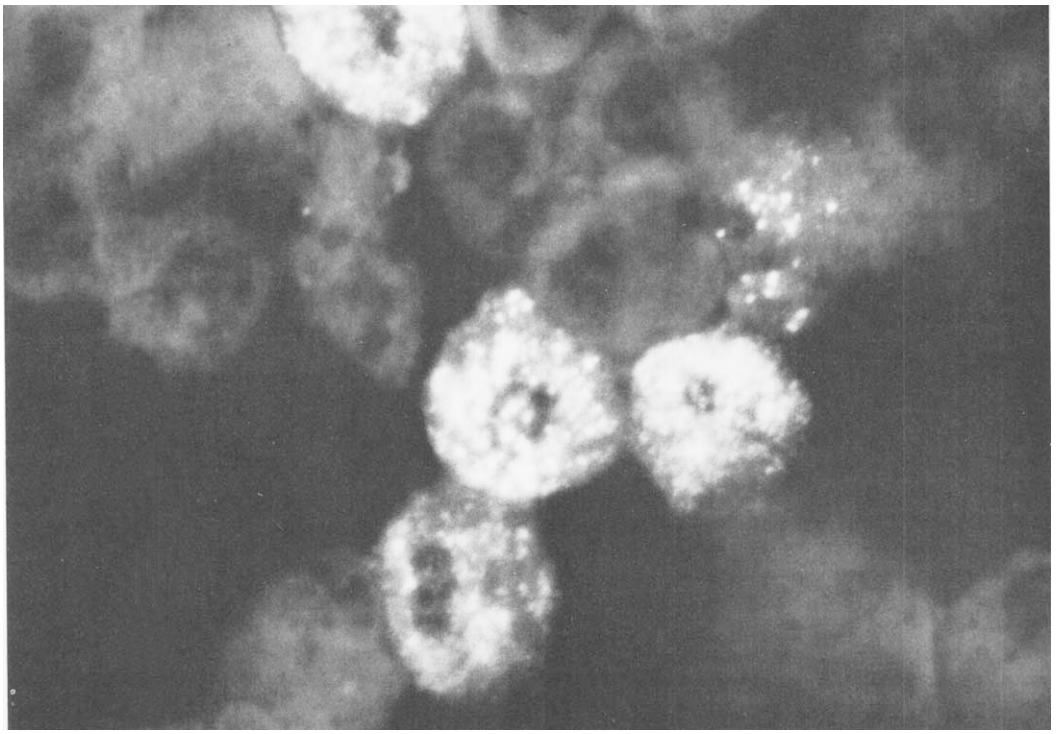

FIG. 4. Localization of rotavirus antigen in the cytoplasm of enterocytes isolated from MRV-infected suckling mice.

fected with the virus at 12–19 days of age and the mean number of virus positive cells was about 10% (Fig. 5). Of particular importance is the observation that very few antigen-positive enterocytes were found in the animals infected with MRV at 20–26 days of age and no antigen-positive cells were detected in animals after 75 days of age (Fig. 5). No staining for virus antigen was observed in age-matched control animals who were not infected with the virus.

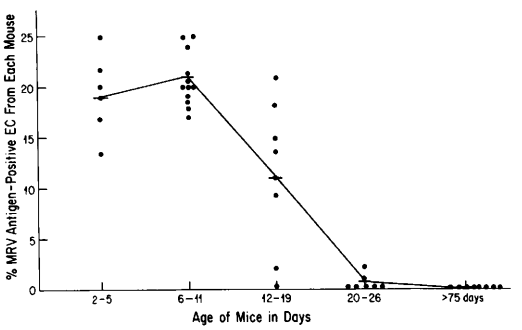


FIG. 5. Percentage MRV-positive enterocytes detected among small intestinal enterocytes isolated from infected and uninfected mice.

In an effort to determine whether enterocytes obtained from susceptible suckling mice could be infected *in vitro,* specimens of enterocytes were obtained from 4- to 7-day-old uninfected suckling mice. The cells were incubated with 0.1 ml of concentrated virus in media 199 with 10% fetal calf serum at 37°. In a few experiments, attempts were made to create a solid phase (enterocytes on plastic or glass surface, employing poly-L-ysine or agarose) to facilitate cellular infection. After 24 hr of incubation, the cells were harvested and examined for the presence of immunofluorescent viral antigen. To date, all attempts to induce MRV infection in isolated enterocytes in *in vitro* settings have failed and no intracytoplasmic or membrane fluorescence for

MRV could be detected in such enterocytes.

*Binding of MRV to enterocytes in vitro.* Isolated enterocytes obtained from uninfected suckling mice produced a very few (1 to 15/2000 enterocytes) rosettes when incubated with control RBC not coated with MRV. Similarly, preincubation of uninfected enterocytes with free MRV prior to incubation with MRV-coated RBC resulted in little or no rosette formation (Table 1). The distribution of enterocyte binding to MRV-coated RBC relative to the age of the mice at the time of collection of enterocytes is presented in Fig. 6. The highest number of enterocytes with MRV–RBC rosettes was found in the suckling mice under 11 days of age. About 250–350 rosettes from 2000 enterocytes were observed in these animals. Subsequently, the number of such enterocytes declined with advancing age of the suckling mice and less than 75–100 virus-binding enterocytes were detected after 75 days of age (Fig. 6). It is interesting to note that the temporal pattern of enterocyte–virus binding *in vitro* was remarkably similar to the patterns of cytoplasmic viral antigen detected after *in vivo* infection of the intact animal. However, no antigen-positive enterocytes were observed in infected animals older than 26 days of age, although small numbers of virus-binding enterocytes *in vitro* could still be detected in this age group of animals as well as in the older animals (after 75 days) as shown in Fig. 6.

**Discussion.** The information presented in this report indicates that replication of mouse rotavirus as evidenced by the detection of fluorescent viral antigen in the cytoplasm occurs in up to 25% of enterocytes after oral infection with the virus. Approximately similar numbers of enterocytes were found to be able to bind to MRV-coated RBC in *in vitro* settings. Of particular importance is the observation that the intracellular replication of the virus was an age-dependent phenomenon. The enterocytes obtained from suckling mice under 11 days of age exhibited most pronounced cell–virus binding and virus replication. Although no virus antigen was detected in the cytoplasm of enterocytes obtained from mice older than 20 days of age, low levels of rotavirus binding activity persisted in the enterocytes from mice older than 75 days of age.

Several recent studies have demonstrated that uptake of protein molecules at the intestinal epithelial level is a specific receptor-dependent phenomenon. For example, the penetration of mouse reovirus has been shown to be restricted in their attachment to the intestinal M cell (17). Specific binding sites have been observed in various enterocytes of neonatal rats for mucosal attachment of the immunoglobulin isotype IgG and secretory IgA (18). Previous studies from our laboratory have suggested possible binding of virus-specific antibody to such sites, based on the fact that approximately 20–30% of orally administered poliovirus antibody in human infants could not be accounted for by its presence in the serum or by fecal elimination (19). Finally, in a series of experi-

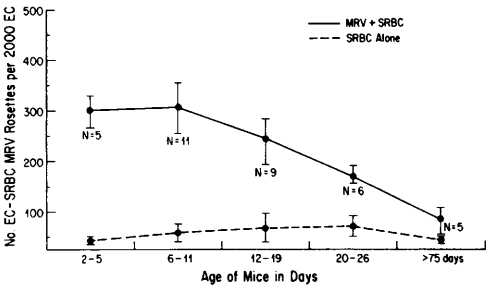


FIG. 6. Number of enterocyte–SRBC$^{MRV}$ rosettes detected among 2000 enterocytes isolated from the small intestine of mice of varying age groups.

TABLE I. CONTROL EXPERIMENTS FOR MOUSE ROTAVIRUS (MRV) ENTEROCYTE (EC) BINDING *IN VITRO*, USING MRV-COATED SHEEP RED BLOOD CELLS (RBC$^{MRV}$) OR CONTROL (UNCOATED) RBC

| Cell suspension | EC–RBC rosettes (% per 2000 EC counted, range) |
|---|---|
| EC + RBC | 0.75–3.7 |
| EC + MRV + RBC | 1.4–3.5 |
| EC + MRV + (RBC$^{MRV}$) | 1.6–3.9 |
| EC + (RBC$^{MRV}$) | 12.5–17.5 |

ments, Morris (20) has provided strong evidence to suggest the presence of Fc receptors in rodent enterocytes which may effect the uptake of IgG in the intestinal epithelium. Significantly, however, such receptors occurred only in the intestine of rats under 17–18 days of age and the disappearance of these receptors could be induced prematurely by treatment with hydrocortisone. In view of the age-dependent cytoplasmic localization of the MRV and its binding to the enterocyte cell membrane, and other observations summarized above, it is suggested that the outcome and the pathogenesis of rotavirus infection in the suckling mouse may be determined by the availability of specific binding sites in the proximal small intestine. It is proposed that the suckling infant less than 11 days of age possesses the highest numbers of such binding sites and these numbers decline significantly after 21 days of age as the animals are weaned.

It is not known whether the process of suckling or the maternal milk is related to induction and subsequent prevention of the binding site in the suckling infant. In the present studies, no data are available on the nature of the binding site. Previously it has been hypothesized that the enzyme lactase which is present in abundance in the intestinal brush border of infant mammals may be the site for virus binding (21). However, this hypothesis has not been adequately tested to date.

Available data indicate striking similarities between the kinetic patterns of immunoglobulin binding in infant gut and replication patterns of rotavirus in intestinal epithelium (5, 7, 11, 20). In view of their anatomic restriction to the proximal small intestine, and the highly predictable age dependance for both phemenon, it is tempting to propose that the binding site for rotavirus may represent Fc receptors similar or identical to these previously shown for immunoglobulins (20, 22).

The implications of the observations reported above should be applicable to the understanding of human and other mammalian rotavirus infections. Human rotavirus appears to result primarily in asymptomatic intestinal infection in the early neonatal period and adults (23, 24) and the peak incidence of clinical disease is limited to infants under 24 months of age. Our data may help to explain the lack of clinical disease in adult mammals but it does not address the lack of disease in the neonate. It also remains to be seen whether the pattern of enterocyte–rotavirus interaction and the replication patterns of rotavirus in human enterocytes are similar to these observed with induced infection in the mouse model.

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