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Effect of Malnutrition on Rotavirus Infection in Suckling Mice: Kinetics of Early Infection[1] (41987)

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Abstract. The effects of malnutrition on the viral replication pattern and severity of clinical disease were examined in suckling mice infected with mouse rotavirus (MRV). The infection in malnourished animals was characterized by a significant decrease in the minimal infectious dose and in the incubation period of the onset of diarrhea, when compared to well-nourished controls. Viral replication in the dispersed enterocytes was observed 6–12 hr earlier, fecal viral shedding peaked significantly earlier, and the clinical disease appeared to be more severe in the malnourished animals than in the controls. These observations provide strong evidence for malnutrition-induced alterations in the pathogenesis of rotaviral infection *in vivo*. © 1985 Society for Experimental Biology and Medicine.

Epidemiologic data suggest a close relationship between malnutrition and development of severe diarrheal disease (1–3). Nutritional deficiencies have been reported to impair immunological response and increase susceptibility to infections (4–6). In poor socioeconomic settings, especially in developing nations, where malnutrition is a major problem, the common viral infections of childhood appear to be more frequent, severe, and prolonged, and gastroenteritis with severe diarrhea and dehydration is a significant problem. Rotavirus is known to be the single most important cause of viral enteritis in infants and children all over the world especially in areas where protein calorie malnutrition (PCM) is frequently seen (7). The two states function together to the detriment of the affected subjects. However, it is not clear as to what extent PCM influences susceptibility of the host to the replication of the infectious agents.

The present investigation was designed to examine the impact of malnutrition on the early events of rotavirus infection in suckling mice. This system was chosen because the pathogenesis and clinical manifestations of rotavirus infection are very similar in suckling mice and human infants (8–10). In addition the murine model lends itself to a manipulation previously employed in suckling rats to induce malnutrition through expansion of litter size (11–13).

Materials and Methods. *Animals and induction of malnutrition.* A total of 350 BALB-C mice (West Seneca Breeding Laboratories, West Seneca, N.Y.) were utilized in these experiments. Infant mice, within 12 to 16 hr after birth, were distributed so that normal litters consisted of 7 to 9 babies and expanded litters consisted of 18 to 20 babies. All litters were maintained in the breeding laboratory and shipped in the same cages the day before virus inoculation, to reduce cannibalism and the risk of exogenous infections other than the experimental rotavirus.

Weights of the babies were taken by the breeding laboratory personnel the day of birth, the day before shipping, and the day of shipping. Quantitative measurement of serum albumin was carried out on pooled sera from 6- and 8-day-old malnourished and normally nourished mice. The Bromcresol Green Albumin Reagent test was used (Worthington Diagnostics Systems, Inc., Freehold, N.J.).

Experimental infection. Mouse rotavirus (MRV) EDIM 5099 supplied by Dr. R. Wyatt, National Institutes of Health (NIH), was passed 14 times in suckling mice. Clari-

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fied, sonicated intestinal homogenate was used as virus inoculum. The 50% infectious dose endpoint of the virus pool was calculated according to the Reed–Muench method. Sixty-seven mice in 10 litters were fed with serial dilutions of virus in a constant volume of 5 μl. The ID_{50}/ml was established as the dose of virus which produced moderate diarrhea in 50% of the animals of a litter, 72 hr after virus ingestion. The titer of the pool used in all the experiments described here was 2×10^7 ID_{50}/ml.

Infection was induced in suckling mice by oral ingestion of various doses of MRV. Animals were observed for the appearance of diarrhea at 12 hr postinoculation thereafter at frequent intervals. The severity of disease following ingestion of the virus was assessed by the appearance of fecal staining in the animals and the interval between its appearance and the ingestion of the virus. It should be pointed out that no fecal staining was observed in well-nourished infected controls. The extent of viral replication was determined by immunofluorescent staining of isolated enterocytes at 24 and 48 hr after inoculation. Fecal virus shedding was monitored by an enzyme-linked immunosorbent assay (ELISA).

Immunofluorescent staining of isolated enterocytes. Epithelial cells were dissociated from the proximal small intestine of infected and uninfected mice according to a procedure previously described (14). After adequate washing, air dried smears were made of the enterocytes. These were fixed in cold (4°C) acetone for 10 min. The fixed cells were stained with fluorescein-conjugated monospecific antibody to rotavirus prepared in gnotobiotic pigs (kindly supplied by E. Bohl and Linda Saif, Ohio Agricultural Research and Development Institute, Wooster, Ohio) washed and read in a microscope equipped with a mercury vapor bulb (Leitz Ortholux, Wetzlar, West Germany). A minimum of 2000 cells was examined from each preparation and a percentage of positive cells per total cells was calculated.

Enzyme-linked immunosorbent assay (ELISA). The indirect enzyme immunoassay adapted from the technique described by Yolken and his colleagues (15) was employed. Briefly, plastic beads coated with guinea pig hyperimmune anti-rotavirus serum, (kindly supplied by Abbott Labs, Chicago, Ill.) were incubated with the fecal samples. After washing the beads with buffer, peroxidase-conjugated rabbit anti-serum to rotavirus was incubated with the coated beads. The unbound material was removed by repeated washing before the addition of (*o*-phenylenediamine 2HCl) as substrate for peroxidase. After 15 min of incubation at room temperature the resulting absorbance was read using a precision spectrophotometer (quantum 1-cm dual wavelength analyzer) at a wavelength at 492 nm.

Data analysis. Student's *t* test was used to determine whether significant differences existed between weights of malnourished and normal animals. The χ^2 was used to estimate differences between time of onset of diarrhea and fecal viral shedding as well as to compare percentage of positive enterocytes from normal and malnourished groups of animals. Differences in serum albumin levels were measured by the Kruskal–Wallis test corrected for samples of three or less. Each of the six serum albumin samples tested represented pooled sera from 8 to 20 mice. The references for the data analyses was *Statistical Methods* by Snedecor and Chochran published by Iowa State Press, Ames, Iowa, 1967.

Results. When suckling mice, 12 to 16 hr old, were distributed and maintained in litters of 18 to 20 babies with a single mother, they were severely protein calorie malnourished (PCM) by 6 days of age. Table I demonstrates that mice in an expanded litter achieved only about 60% of the weight of mice in a normal litter at 4 through 8 days of age. In addition the serum albumin levels were 14% less in malnourished mice at 6 days after birth (Table II). Both the differences in weights and serum albumin levels were significant $P < 0.001$.

TABLE I. COMPARISON OF WEIGHTS[a] IN MALNOURISHED AND CONTROL MICE

Day after birth	Normal litters	Expanded litters
1	1.4 ± 0.1 (28)	1.5 ± 0.2 (70)
4	3.6 ± 0.2 (33)	2.1 ± 0.3 (77)
6	3.8 ± 0.3 (20)	2.3 ± 0.2 (36)
8	5.3 ± 0.2 (20)	3.3 ± 0.3 (30)

[a] Weight in grams ± SD (*N*).

TABLE II. COMPARISON OF SERUM ALBUMIN LEVELS[a]

Day after birth	Normal litters[b]	Expanded litters[b]
6	1813 ± 357	1576 ± 228
8	1907 ± 292	1706 ± 39

[a] mg/dl ± SD.
[b] Sera of two litters were pooled for each of three test samples at Days 6 and 8.

Following inoculation of varying doses of the virus ($1 \times 10^{0.1}$ to 1×10^5 ID_{50}), malnourished animals exhibited the appearance of diarrhea at a significantly earlier time ($P < 0.001$) than those observed in the well-nourished controls (Table III). As shown in the table, diarrhea appeared 25–30 hrs earlier in the malnourished animals than in the controls at every dose of the virus employed. In addition, diarrhea appeared to be more severe as evidenced by the appearance of fecal staining with all doses of the virus in the malnourished animals compared to the lack of staining in the controls.

The clinical parameters of severe MRV infection in the malnourished animals were supported by the degree of fecal viral shedding shown in Fig. 1. While virus was detected in the feces 24 hr after every infecting dose in malnourished animals, it was only barely detectable at 10^2 ID_{50} in normally nourished controls. Furthermore higher fecal viral shedding was observed in malnourished animals with an infecting dose of 1×10^5 ID_{50}. Therefore subsequent data on the kinetics of rotavirus infection were obtained after using 1×10^5 ID_{50} as the infecting dose for both groups of animals.

MRV antigen was detected in dispersed enterocytes as early as 12 hr after infection in the malnourished animals and the peak number of infected enterocytes was attained by 15 hr. However, only occasional infected enterocytes were observed after 24 hr in the malnourished animals (Fig. 2). On the other hand, control animals exhibited infected enterocytes in the gut after 15 hr of infection and the proportion of infected enterocytes peaked 24–48 hr after infection. There were significant differences in the numbers of infected enterocytes from malnourished and normal animals detected at 15 and 24 hr after infection ($P < 0.001$). Although the frequency of infected enterocytes declined shortly in the control animals as well, a few (4%) MRV antigen positive enterocytes could still be detected in the controls for as long as 48 hr after infection.

The temporal pattern of fecal viral shedding after inoculation of 1×10^5 ID_{50} dose of the virus is shown in Fig. 3. Malnourished animals were found to shed virus in the feces 12 hr after infection, significantly earlier than the controls $P < 0.001$. The level of shedding peaked by 18 hr and persisted at high levels for as long as 48 hr. On the other hand, fecal viral shedding in the controls appeared 12 hr later than in the malnourished animals and the peak viral shedding was not observed until 48 hr after infection.

TABLE III. CHARACTERISTICS OF DIARRHEA IN ROTAVIRUS-INFECTED MALNOURISHED AND NORMAL MICE

Virus dose (ID_{50})	Onset of diarrhea (hours after ingestion)[a] Malnourished (N = 119)	Normal (N = 68)	P[b]	Severity of diarrhea[c]
10^5	21.5 ± 1.5	46 ± 2	<0.001	25.5 ± 2.5
10^2	25.5 ± 1.5	50 ± 2	<0.001	39 ± 3
10^1	26 ± 2	50 ± 2	<0.001	39 ± 3
$10^{0.1}$	31.5 ± 4.5	60 ± 12	<0.001	46 ± 2

[a] Mean ± SD.
[b] Based on χ^2.
[c] Based on fecal staining observed hours after ingestion in malnourished mice. No staining observed in normal mice.

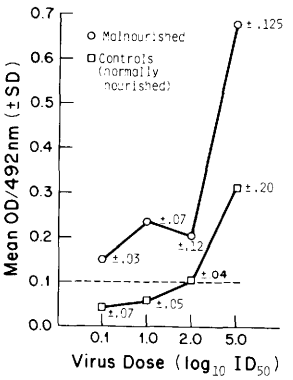

FIG. 1. Effect of altered nutrition on appearance of rotavirus in feces (positive above serated line).

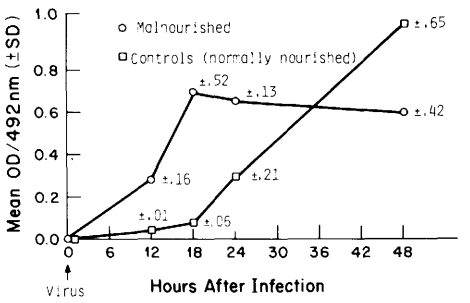

FIG. 3. Kinetics of appearance of rotavirus antigen in feces (positive above 0.1 OD).

Discussion. The observations of particular importance in the present report are the significant reduction in the incubation period and in the minimal dose of virus necessary for expression of rotavirus-induced diarrhea in malnourished animals. Recently, a prospective epidemiologic study of 97 adults and children suffering from cholera has suggested that a high proportion of infected subjects with second degree malnutrition exhibits prolonged diarrhea (16). Few studies have been carried out which characterize the relationship between the severity of infectious enteritis and the preexistence of PCM. To date, little or no information is available regarding the mechanisms underlying the evolution of severe diarrhea in patients with moderate to marked nutritional deficits.

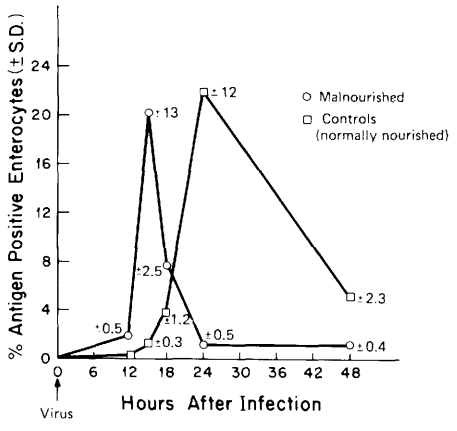

FIG. 2. Kinetics of appearance of rotavirus antigen in enterocytes.

The studies reported here have utilized the murine model and have compared rotavirus-induced diarrhea in normally nourished suckling mice and in suckling mice maintained in expanded litters. Other studies have shown that undernutrition during the suckling period results in permanent morphological and metabolic changes in mice and rats. Hatch and colleagues (11) using litter expansion for 19 days induced a severe state of malnutrition as evidenced by substantially decreased total body weight, and intestinal wet weight in malnourished rats compared to normals. They determined that this reduction was due to impaired cellular multiplication based on a significant reduction in the total DNA content in the small intestine of malnourished rats, in proportion to the diminution in total intestinal wet weight and total body weight. These changes in the intestine were similar to those described in other organs namely brain, heart, liver, kidney, thymus, and spleen in 21-day-old rats who had been placed in an expanded litter at birth (12, 13, 17, 18).

It has been demonstrated in mice and in other mammalian species that the replication of rotavirus is exquisitely limited to the terminal epithelial cells of the small intestinal villi. The differentiated crypt cells do not appear to support viral replication (19). In the present studies, the replication of virus in the enterocytes appeared to peak within 15 hr and essentially no antigen positive enterocytes could be observed after 24 hr of high dose infection in malnourished animals. This is in striking contrast to the observations in well-nourished animals in whom antigen containing enterocytes were detected 10–12

hr later and the continued presence of such enterocytes was observed for as long as 48 hr (Fig. 2).

While it appears that appreciable numbers of infected enterocytes were not evident after 24 hr, it should be kept in mind that these studies have characterized the kinetics of infection after a large virus dose for the first 48 hr after virus infection only, and have not defined events after that time. In normal mice we have seen from previous studies (20) that there appeared to be recruitment of new enterocytes possibly farther along in the intestine and at 72 hr after virus ingestion the number of infected enterocytes began to rise again only to drop off finally at 4 to 6 days postinoculation. It remains to be seen whether the gut of malnourished mice is capable of additional virus replication after the first round.

The appearance of rotavirus in the feces based on increasing inoculum size (Fig. 1) demonstrates that in malnourished mice a significantly smaller virus dose produces active replication and detectable virus shedding. On the basis of these observations it is proposed that malnutrition may reduce the threshold for the minimal infectious dose of the virus, possibly because the altered enterocyte membrane presents less barrier to viral penetration.

The kinetics of antigen in the feces mimics that of enterocyte associated antigen but it is more protracted. While antigen is detectable in feces from both normal and malnourished animals, the levels appeared to be significantly lower from normally nourished animals during the first 24 hr. More rapid viral penetration in the malnourished mice may be the result of greater permeability of the enterocyte membrane. Rothman and colleagues have shown that there is significantly increased uptake of macromolecules in the intestine of malnourished 1-month-old rats compared to normal rats (21). Alternatively, some attention must be given to the fact that individuals in expanded litters suckle less than their normal counterparts and therefore receive less anti-rotavirus secretory IgA and other protective factors. However, it is evident based on the data presented here that a single large dose of rotavirus easily contains sufficient numbers of nonneutralized infectious virus particles to initiate infection in recently suckled normal mice and it would appear that the magnitude of the inoculum ensures that the infection is begun as rapidly as possible in normal enterocytes. Thus, it is suggested that the acceleration in rotavirus replicative events in malnourished animals may be due to a specific alteration in susceptible enterocyte cell membrane as a direct result of nutritional deprivation. The precise mechanism of such an alteration remains to be determined.

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