PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 176, 77–83 (1984)

Effects of Folic Acid Malnutrition on Rotaviral Infection in Mice[1] (41845)

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Abstract. A study was undertaken to determine if dietary deficiencies of folic acid would influence rotaviral diarrheal disease in infant mice. Female mice were fed diets containing essentially no folic acid, 25% of a normal quantity of folic acid, or a normally recommended quantity of folic acid, beginning at time of breeding and continuing through periods of gestation and lactation. Two-day-old infants from these dams were exposed to purified murine rotavirus or to sterile virus diluent and the severity of the rotaviral infection monitored. Infants from the low folic acid group had significantly lower folate levels in their livers, indicating a deficiency was achieved, and developed more severe disease manifestations than those infants from the dams receiving the normal folic acid levels in their diet. The infection enhancement was seen as increased incidences of diarrhea and a significantly greater number of mice exhibiting high intestinal rotaviral antigen titers. Serum rotavirus antibody titers were below detectable levels in a significant number of these same infants.

A close association exists between diarrhea and malnutrition, with malnourished children having a higher incidence of severe diarrhea resulting in increased mortality (1, 2). A substantial number of the total episodes of diarrhea have been attributed to rotavirus infection (1).

Noble *et al.* (3) demonstrated in infant mice that general malnutrition, as established by nutrient dilution, and protein deficiency dramatically enhanced the severity of the murine rotaviral disease. Conceptually, some specific nutrients may be responsible for the adverse effect of malnutrition on rotaviral diarrhea, since it is known that deficiencies of certain specific nutrients profoundly affect immunologic response (4, 5). Among such nutrients affecting host immune response is folic acid, which is one of the first limiting nutrients in human diets (6). Folate deficiencies in mice have been shown to reduce the number of plaque-forming units in the spleen and severely impair hemagglutinin antibody in response to red blood cell challenge. Cell-mediated immunity is also reduced as seen by reduction in dermal sensitivity response of phytohemagglutinin, and decreased T-cell population in peripheral blood and spleen (5). The present study investigated the combined pre- and postnatal effects of diets deficient in folic acid on rotaviral disease induced in infants born to these dams.

Materials and Methods. *Diet.* The amounts of folic acid in three different diets were determined using data of the National Academy of Sciences (7). Folic acid in concentrations of 0, 0.125, and 0.50 μg were added per gram of basal diet to make, respectively, deficient, marginal and normal folic acid diets as recommended for normal growth. The basal diet contained (g/kg diet): casein 150, corn oil 60, mineral mix (3) 11.6, vitamin mixture (3) prepared without folate 20, $CaCO_3$ 16.75, $NaHPO_4 \cdot H_2O$ 29.8, cellulose 50, corn syrup 50, and dextrose to make 1 kg. Diets were pelleted. Both food and distilled water were provided *ad libitum.*

Mice. Specific pathogen-free Swiss–Webster mice used in this study were designated Crl: CFW (SW) BR, (Charles River, Wilmington, Mass.), and had been previously found to be quite susceptible to murine rotavirus infection (3). Immediately upon arrival of the adult mice, precautions were taken to not inadvertently expose the mice to rotavirus by placing them in a limited-access, disinfected room. The mice were housed in disposable cages with

[1] Supported by the Thrasher Research Fund and the Utah Agricultural Experiment Station. Utah Agricultural Experiment Station Publication No. 2886.

0037-9727/84 $1.50

filter tops (Lab Products, Inc., Federalsburg, Md.); bedding was changed frequently to avoid coprophagy. Mice infected with rotavirus were kept in a building separate from the housing of the uninfected mice and personnel working with these infected animals were denied access to rotavirus-free areas.

Virus. Murine rotavirus (MRV) was obtained from Dr. M. Collins of Microbiological Associates Inc. (Bethesda, Md.). The virus had RNA band patterns similar to the bovine and human rotaviruses (8), verifying that it was a rotavirus. Stock virus was prepared by inoculating mice by oral gavage (po) with MRV, then harvesting the intestines 4 days later. These were homogenized to a 10% w/v suspension of 0.01 *M* phosphate-buffered saline (PBS), pH 7.2, which was then Freon-extracted and purified further by centrifugation in a cesium chloride density gradient. The virus was collected at a density of 1.3 to 1.4 g/ml and dialyzed against 0.01 *M* PBS, pH 7.2. The virus stock, from which no bacteria could be isolated, was titered in infant mice to determine the 50% infectious dose (ID_{50}); an approximate 100 ID_{50} viral inoculum was used in these studies.

Experimental design. Two sexually mature females were bred with one male. The mice were randomized to the diets by alternately assigning a diet to each mouse. At least 25 female mice were fed each diet group, beginning at the time of introduction of the male and continuing through 24 days after birth of their young. At 2 days of age, 50% of their infants were infected po with rotavirus and the remainder were sham-infected with PBS. Sham-infected mice were housed separately from the infected animals. The presence of diarrhea in the infant mice was measured on Days 1–5, 10, and 20 post-virus infection by slightly pressing the abdomen of the infant and observing for the presence or absence of a highly conspicuous, copious diarrhea. Each litter was weighed on Days 1–5, 10, and 20 post-virus infection.

Intestines from 25–30 infants from each diet group were collected to assay virus titer on Day 4 post-virus infection. The intestines were excised at the pyloric valve to the anus, diluted 1:10 in PBS, pH 7.2, homogenized, and assayed for rotavirus by ELISA. Portions of the duodenum, ileum, and jejunum were also collected and placed in Formalin at 20 days post-virus infection. The Formalin-fixed intestines were subsequently imbedded in paraffin, sectioned, and stained with hematoxylin–eosin stain for histological examination.

Stomachs from infants were removed on Day 4, and serum was collected on Day 20 post-virus infection. At least 20 stomachs from each diet group were processed for antibody assays by homogenizing the curdled milk contents in a 1:5 dilution of PBS. Livers were excised from infant mice at 1 and 4 days post-virus infection, homogenized in a tissue grinder, and assayed for folic acid.

Upon collection of the tissues, all specimens were stored at −20°C. The assays were done soon after the collection of the sample.

Folic acid assay. The liver folic acid levels were assayed by the method of Bennett *et al.* (9) by placing a portion of homogenized liver in media without folic acid to a standardized concentration of 10% (w/v). Commercially prepared Folic Acid Casei Medium was used (Difco Laboratories, Detroit, Mich.). *Lactobacillus casei* ATCC 7469, which depends on extraneous folic acid for growth, was seeded to the media. The degree of growth, as measured by absorbance at 670 nm, was proportionate to the amount of folic acid in the liver. A standard curve was calculated to quantitate the folic acid in the liver.

Titration of viral antigen. Viral antigen in intestinal homogenates was assayed by ELISA (10). The test was performed by first filling 96-well microtiter plates (Polyvinyl V-well, Dynatech, Alexandria, Va.) with ammonium sulfate precipitated guinea pig anti-simean rotavirus immunoglobulins, and then incubating for 18 hr at 4°C. The wells were washed nine times for 30 sec each with distilled water. All subsequent washings were the same. Serial dilutions of the samples in Brain Heart Infusion Broth (BHI, BBL Microbiology Systems, Cockeysville, Md.) were added to the wells and incubated overnight at 4°C. After washing the wells again, rabbit anti-human rotavirus peroxidase conjugate (DAKO, Accurate Chemicals, Westbury, N.Y.) containing 20% fetal bovine serum and 2% normal guinea pig serum was added to the wells and incubated 3 hr at 37°C. After washing, Rotazyme sub-

strate (Abbott Laboratories, North Chicago, Ill.) was added and incubated 30 min at room temperature. After stopping the reaction with 1 *N* HCl, the absorbance at 490 nm was read on a Microelisa reader MR 590 (Dynatech Laboratories, Inc., Alexandria, Va.). Titers were expressed as the highest dilution giving a $P/N > 2.1$, where P is the absorbance at 490 nm of the sample and N is the average of six wells containing rotavirus-negative intestinal homogenate.

Titration of serum and stomach milk antibody. Milk and serum anti-rotavirus antibodies were assayed using blocking of bovine rotavirus (BRV) detection by ELISA (11). Equal volumes of Freon-extracted cell culture-propagated BRV and varying twofold dilutions of the mouse serum, or milk to be tested for anti-rotavirus antibody were incubated together at 37°C. After 1 hr incubation, titration of bovine rotavirus in these mixtures was carried out as described above for titration of viral antigen. A 50% reduction in absorbance at 490 nm between the mean of three positive controls containing no sera compared to the mean of three negative controls (BHI) was considered positive for rotaviral-specific antibody. This 50% reduction end point was as described by Yolken *et al.* (11). The end point of the sample being tested was the highest dilution that was positive for antibody.

Statistical analysis. The percentage weight gain data were analyzed by expressing the experimental design in the form of a model, $y = I_i + D_j + C_{(ij)k} + T_l + ID_{ij} + IT_{il} + DT_{jl} + IDT_{ijl} + E$, where I was infected or sham-infected, D was diet, C was cages and T was days after inoculation. The analysis of variance was computed by RUMMAGE program (Bryce, G. R. Data Analysis in Rummage—A User's Guide. Brigham Young University, Provo, Utah).

Means for antigen and antibody titers were calculated by obtaining the $\log_{10}$ of the inverse of each titer, averaging those values, obtaining the antilog of that mean, and obtaining the inverse to arrive at the mean for the antigen or antibody titers. The data for serum antibodies, intestinal antigen, and presence of diarrhea were analyzed by chi-square analysis using Yate's correction for small sample size.

Chi-square analysis for antibody data was performed by obtaining the mean of all the data, and using the mean value as a division line; those values above the mean were connoted (+) and those below were connoted (−) for use in the statistical analysis.

Results. The folic acid contents of livers from infant mice used in this study are summarized in Table I. Infants from mice receiving 0 μg folic acid/g diet had reduced quantities of folate in their livers, indicating the diet was sufficient to achieve a folic acid deficiency in the animal. Mice that received the higher dietary levels of folic acid exhibited no apparent decrease of folate in their livers.

The results of the diet/infection study are summarized in Table II. The sham-infected mice from dams receiving the lowest folic acid diet gained less weight ($P < 0.05$) than the sham-infected mice from the dams fed the two diets having higher levels of folic acid. The sham-infected mice fed the marginal folic acid diet appeared also to gain less weight than did the mice receiving the highest folic acid diet; the difference, however, was not statistically significant. More obvious in Table II is the substantially reduced weight gains of the rotaviral infected infants compared with those of the uninfected infants ($P < 0.001$, when compared to uninfected controls). Although infected infants from mice fed the 0 μg folic acid/g diet had the overall lowest weight gains, the differences as compared with the infected animals in the higher folic acid diet groups were not statistically significant.

The proportion of mice exhibiting profuse diarrhea on Days 2–5 post-virus inoculation are also seen in Table II. The diarrhea had totally subsided in all groups by Day 10. Mod-

TABLE I. FOLIC ACID CONTENT[a] OF LIVERS FROM UNINFECTED INFANT MICE OF EACH FOLIC ACID DIET

Age of mice	Diet[b]		
	0	0.125	0.50
3 Days	3.8 ± 1.5[c]	5.1 ± 1.5	5.3 ± 0.9
6 Days	3.7 ± 0.9	4.5 ± 1.0	6.2 ± 1.2

[a] Micrograms of folate per gram of liver.
[b] Micrograms of folate per gram of basal diet.
[c] Mean ± standard deviation. From left to right the number of samples per diet group assayed were 6, 3, 4, and 4, 13, 3.

TABLE II. EFFECT OF PRE- AND POSTNATAL DIETARY FOLIC ACID DEFICIENCIES ON ROTAVIRUS INFECTION IN INFANT MICE

	Folic acid concentration in diet[a] (μg/g)					
	0		0.125		0.5 (Control)	
	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected
No. of mice	136	138	91	60	85	68
Av initial wt (g)	2.1	2.0	2.1	2.0	2.2	2.0
Mortality (%)	0	0	2	0	0	0
Day 20 wt gain (%)	400	520*	442	542	430	595
Total incidence of diarrhea[b] (%)	61**	0	48	0	50	0
Mean intestinal viral titer[c]	544 (180–1640)[e]	0	740 (266–2058)	0	386 (206–724)	0
Mean rotaviral antibody titer[d] (dams' sera)	10 (6.2–17.4)	0	9 (3.5–24.5)	0	13 (7.2–22.4)	0
Mean rotaviral antibody titer[d] (infants' sera)	2.4 (1.6–3.8)	0	3.7 (2.0–6.8)	0	3.2 (1.8–5.6)	0

[a] Diets of dams from which infants were taken.
[b] Days 2–5 post-virus exposure.
[c] Titers expressed as reciprocals. Intestines taken 4 days post-virus exposure.
[d] Titers expressed as reciprocals. Sera taken 20 days post-virus exposure.
[e] Standard error.
* $P < 0.05$, ** $P < 0.01$ (compared with controls).

erate ($P < 0.05$) increases in incidence of diarrhea were seen, particularly on Day 2 in the infants from the lowest folic acid dietary group (76% compared to 60% in the mice from the highest folic acid diet group). These differences were still seen on Day 3, but had appreciably declined in all groups by Day 4.

The individual rotaviral antigen titers in intestines from infected infants killed on Day 4 are indicated in Fig. 1. Mean antigen titers, seen in Table II, were increased in the mice from the folate-deprived groups. Although these increased titers were not statistically significant, it was quite apparent that all intestines from the control group had lower titers (none >640) than those from the folate deficient groups. By utilizing chi-square analysis where those titer values greater than or equal to 1280 were connoted (+) and those below 1280 were connoted (−), the number having high titers in the lowest and marginal folic acid diet groups were significantly increased ($P < 0.05$) compared with the control (0.5 μg/g) folic acid diet group, indicating the folate deficient diets may have enhanced the MRV infection in a significant number of the infants. No serum rotaviral antibody or intestinal rotavirus was found in uninfected dams and infants.

A small degree of cytoplasmic vacuolization in the epithelial cells at the tip of the villi was seen in infected mouse intestines from all three dietary groups. There was no difference in the severity of this degeneration between any of the three dietary groups.

Milk in the stomachs of infected infants at 4 days post-virus infection was assayed for rotaviral-specific antibody, but no antibodies were detected at the lowest dilution (1:5) tested. Serum rotaviral antibody titers of infected dams and infants from each dietary group killed on day 20 are summarized in Table II, and individual antibody titers for the infants' sera are seen in Fig. 2. Sera from infants in the lowest folic acid dietary group had

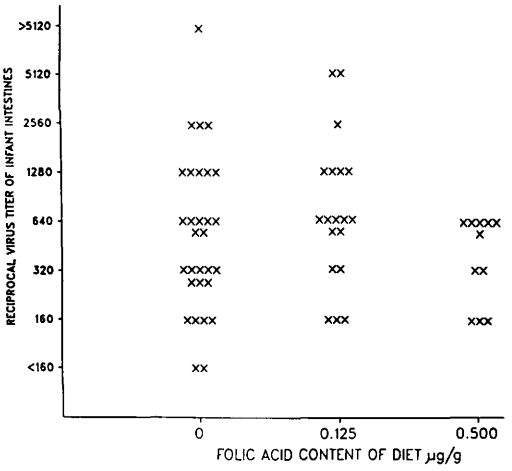

FIG. 1. Reciprocal virus titers in intestines from infected infant mice 4 days post-virus inoculation whose dams received pre- and postnatally 0, 0.125, and 0.5 μg/g folic acid diets.

overall lower titers than did those from either marginal or normal folic acid groups. A significant number ($P < 0.05$) had titers of <1:4. The rotaviral antibody titer of the dams' sera was not appreciably altered in any group. These animals were not directly infected with rotavirus, but were presumably exposed to the virus by being in close proximity to their experimentally infected infants.

Discussion. Varying levels of folic acid in a basal diet were fed to female mice to cause reduced or normal folic acid levels in their progeny. A folic acid deficiency was observed in the infant mice born to dams fed the most deficient diet as evidenced by the reduced concentrations of folic acid in the infants' livers. It is noteworthy to compare the values of the folic acid concentrations in the livers with the studies of Klipstein and Lipton (12). They observed the normal range of folic acid in mouse livers to be 6.0 to 13.4 μg/g (μg folic acid/g wet liver) and the range for deficient animals to be below 5.3 μg/g. We also observed similar values of 5.3 to 6.2 μg/g and 3.7 to 5.1 μg/g for the respective normal and deficient folic acid liver concentrations. The folic acid deficiency observed in our study was not believed to be severe, since slight weight retardation was the only clinical manifestation of the deficiency.

The rotaviral disease was moderately enhanced in infants from the deficient and marginal folic acid diet groups. This was apparent by the increased rotaviral titers of the infant mice in these folate deficient groups, and in an increased proportion of mice with diarrhea which was most pronounced early in the infection, i.e., Days 2 and 3 post-virus exposure. The diarrhea, when occurring, was readily apparent as a profuse yellow liquid. The increased incidence of diarrhea may have been a direct manifestation of the increased virus present in the intestine. It is surprising that despite this increased diarrheal incidence in the folate-deprived mice, the weight gain was not influenced to a greater degree than was observed (maximum of 30% reduction by Day 20). It was previously reported by Noble *et al.* (3) that general malnutrition or protein deprivation increased the severity of the rotaviral disease to the point of significant numbers of mice dying of the infection. Such deaths were not seen in the present study, and indeed are an unusual occurrence in experimentally induced rotaviral infections in mice. One change in protocol, however, between the study of Noble *et al.* and the present study was our use of a more purified virus, which may have been less capable of killing the mice.

As was also observed by Noble *et al.* (3), we found that lessened weight gain of the in-

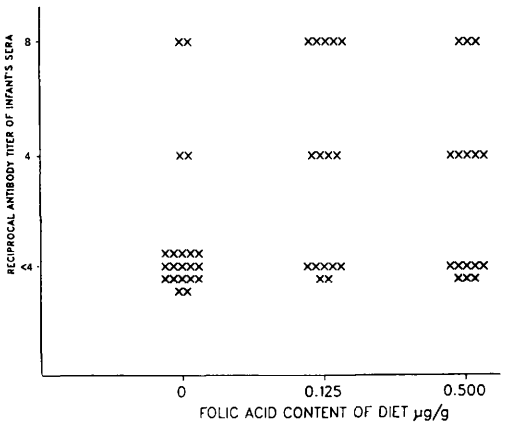

FIG. 2. Reciprocal rotaviral antibody titers in sera from infected infant mice 20 days post-virus inoculation whose dams received pre- and postnatally 0, 0.125, and 0.5 μg/g folic acid diets.

fant mouse is a good parameter to measure the severity of the rotaviral disease. Such failure to gain weight is probably due to diarrhea-induced dehydration. Using this weight gain parameter, it was determined that the infection was apparently of sufficient magnitude to inhibit weight gain in 20 days by over 150%.

Folic acid deficiencies have been reported to affect disease status in several studies involving microbial infections. Nelson *et al.* (13) observed that infant guinea pigs fed a folate-deficient diet for 2 weeks were highly sensitive to infection with *Shigella flexneri,* with 89% of the animals dying after challenged compared to no deaths occurring in similar animals fed a normal diet. Williams *et al.* (14) reported that rats deprived of folic acid post-weaning had a decreased resistance to infection with *Salmonella typhimurium.* Similarly folate-deprived rats have also been found to develop higher and more prolonged parasitemias when infected with *Trypanosoma lewisi* (15). Chicks from a Newcastle disease virus-vaccinated flock which were raised on a folate deficient diet were reportedly markedly more susceptible to later lethal infection with the virus (16). In all these studies, a considerable deranged immunocompetence was noted in the folate-deprived animals.

It was noted in the present study that serum rotaviral specific antibody titers in the folic acid deficient infant mice were lower than the titers of the control mice. This effect was likely due to the reduced folic acid levels and is not surprising when one considers the central role of folic acid in cellular DNA synthesis (17). Among the effects on humoral immunologic response brought about by folic acid deficiency reported by others are decrease in hemagglutination titers (18), decrease in number of antibody-forming cells (19) and decreased bacterial agglutinating antibody titers (16). We saw no significant effect in this study on the folic acid-deprived dams' serum rotaviral antibody titers, but all the titers were quite low and perhaps the time of serum collection was not ideal for achievement of maximal antibody levels. The dams were not directly infected, but presumably became infected from the diarrheic discharge of their infants. The failure to detect maternal antibody in the milk was probably because the sampling time (4 days) was so soon after virus exposure. Other studies being run in our laboratories (B. Barnett, personal communication) have demonstrated that immunized dams excrete high titers of rotaviral antibody in their milk which can be readily assayed by the method described in this report.

The results of this study provide additional insight into the role the water-soluble vitamin, folic acid, may play in the resistance of a host to virus infection.

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Received September 26, 1983. P.S.E.B.M. 1984. Vol. 176.
Accepted February 13, 1984.