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CARRAGEENAN IN FORMULA AND INFANT BABOON DEVELOPMENT

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Male and female infant baboons were reared from birth to 112 days of age on infant formulas containing concentrations of carrageenan varying from none to 5 times the concentration in commercially available formulas for human infants. Carrageenan content of the formula did not affect weight, characteristics of urine and feces, findings on physical examination, hematological variables, blood chemical analyses, organ system weights, or the macroscopic and microscopic appearance of the gastrointestinal tract.

Carrageenan, a sulfated polysaccharide extracted from various species of red algae, long has been used as a component of foods. Currently, it is added to many products to stabilize suspensions of proteins, particularly milk proteins, in such foods as ice cream, chocolate milk, and processed cheeses. Initial tests[1,2] of native (high molecular weight) carrageenan, which is used in foods, showed no adverse effects on rats or mice except at very high concentrations in the diet. Degraded (hydrolyzed, low molecular weight) carrageenan is not used in foods.

Between 1969 and 1974, several reports[3-17] of the effects of both undegraded (native) and degraded (hydrolyzed) carrageenan on the alimentary tract appeared. In brief, these reports indicated that adding degraded carrageenan to the drinking water produced lesions of the colon and rectum, varying from minimal mucosal changes to ulcerations and squamous metaplasia, in rats, mice, rabbits, guinea pigs, and rhesus monkeys. Four reports[4,7,9,15] indicated that colonic ulcerations also were produced in guinea pigs fed undegraded carrageenan. Five reports[3,8,13,16,17] indicated no adverse effects of undegraded carrageenan. Three additional studies,[18-20] concerned with the mechanism of the effect on the colonic mucosa, found that degraded carrageenan (but not native carrageenan) was taken up by, and stored in, the lysosomes of macrophages of the colonic mucosa, and also in the Kupffer cells of the liver.

Because native, undegraded carrageenan is widely used in proprietary infant formulas, it appeared prudent to test formulas made with and without this stabilizer in a nonhuman primate infant. We reasoned that this model would provide the most sensitive and human-like situation in which to detect deleterious effects of native carrageenan on overall growth and development as well as on the alimentary tract and other tissues. Furthermore, the carrageenan would be supplied to the animal in the same form as in formula consumed by humans, that is, heated in the formula and bound to protein to stabilize the complex suspension of fat, protein, and other nutrients.

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Materials and Methods

Experimental animals. The subjects were 24 newborn baboons (*Papio cynocephalus*) from the breeding colony of the Southwest Foundation for Research and Education (SFRE), delivered between May 11, 1973, and January 2, 1974. Insofar as possible, consecutive animals delivered from a colony of approximately 100 females and 6 males were selected.

The newborn infants were taken from their mothers between 18 and 24 hr after delivery and were placed in the SFRE nursery. During the short period with their mothers, most of them had begun to nurse, and had received some colostrum.

Experimental design. The animals were assigned to one of three formula groups according to two random assignment schedules, one schedule for each sex. Because more females than males were born during the experimental period, it was necessary to use 9 males (3 to each diet) and 15 females (5 to each diet).

Feeding and nursery care. In the nursery, the attendant fed each infant baboon five times per day for the first 14 days; four times for the next 14 days; three times for the next 56 days; and two times for the 28 days until 112 days of age. No other fluid or food was provided. The attendant held the animal during each feeding period and offered the animal all of the formula that it would take readily from a freshly opened 120-ml disposable unit with a new sterile nipple. Unconsumed formula in each 120-ml unit was discarded. Formula intake was estimated as the difference between the weight of the bottle before and after feeding.

A veterinarian performed complete physical examinations at the time the infants were received in the nursery and at monthly intervals thereafter. The nursery attendant daily observed the color, amount, consistency, and presence of mucus or blood in the stools, and daily observed the color and approximate volume of urine. Stools were tested weekly for

occult blood by the Hemoccult (Smith Kline Diagnostics, Pennsauken, N. J.) test.

Formula. The three experimental formulas were prepared in the pilot plant of Ross Laboratories (Columbus, Ohio), using procedures that approximated as closely as possible those used in commercial production of infant formulas. Each product was sterilized in 120-ml bottles identical to those used in the commercial production of ready-to-feed infant formulas. Except for the amount of carrageenan, the formulation and preparation of the three products were identical and contained the following ingredients (in decreasing order of predominance in each product): water (Columbus water supply contains about 1 ppm of fluoride), nonfat milk, lactose, soy oil, coconut oil, corn oil, mono- and diglycerides, soy lecithin, ascorbic acid, ferrous sulfate, nicotinamide, α-tocopheryl acetate, potassium citrate, vitamin A palmitate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, pyridoxine hydrochloride, riboflavin, folic acid, vitamin D_3 concentrate, and cyanocobalamin. The nonfat dry milk was taken from a single lot.

The formula composition was not identical with that of any infant formula on the market at the time of the study, but was similar to most of them except in carrageenan content as described below. Each formula was batched to provide about 670 kcal per liter and nutrients as indicated under "basic formula" in table 1. The sterility of each batch also was established by conventional microbiological methods.

To one-third of the formula, no carrageenan was added; to one-third, 300 mg of native carrageenan per liter were added, an amount equal to the highest level used in commercially available formulas for human infants; and to the remaining one-third, 1500 mg of native carrageenan per liter were added. The food grade native carrageenan was SeaKem 2, lot no. 302212 supplied by Marine Colloids, Inc. (Rockland, Me.). The molecular weight distribution of lot 302212, determined by sedimentation equilibrium in the ultracentrifuge by the method of Scholte,[22] ranged from 197,100 to 394,000 with a maximum frequency of 280,000.

The formulas were prepared every 2 months, shipped to San Antonio, Texas, by motor freight, and stored at room temperature. Summaries of analytical results on the batches of formula used are shown in table 1 for each carrageenan level.

Growth and development. Growth was assessed by total body weight measured daily and organ weights at autopsy.

Hematology. At birth, monthly, and before autopsy, blood samples were taken by heel stick for the microdetermination of erythrocytes, leukocytes, red cell indices, and hematocrit utilizing the Coulter model S automated cell counter (Coulter Electronics, Inc., Hialeah, Fla.). Duplicate samples were obtained in most instances. Blood smears were stained with the Ames Hematek slide stainer (Ames Division, Miles Laboratories, Elkhart, Ind.) for differential counting of leukocytes based on 100 cells.

Blood chemistry. Blood was collected just before autopsy in red top Vacutainer tubes (Becton-Dickinson & Co., Rutherford, N. J.) for analysis by the SMA 12-60 AutoAnalyzer (Technicon Instruments Corporation, Tarrytown, N. Y.). Serum protein fractions were determined by electrophoretic separation and densitometric scanning.

Autopsy. We autopsied each baboon within 6 days of the planned 112 day (16 week) experimental period. On the night

TABLE 1. *Target values of nutrients per liter in basic formula and means of results of chemical analyses of five batches of three experimental formulas with standard deviations (in parentheses)*

Nutrient	Unit	Basic formula	Experimental formulas by carrageenan content		
			None	300 mg/liter	1500 mg/liter
Protein (N × 6.38)	g	16.0	16.8 (0.4)	16.8 (0.1)	16.6 (0.2)
Fat	g	36.0	36.5 (0.4)	36.6 (0.4)	35.9 (0.6)
Carbohydrate	g	71.0			
Total solids	g	127.0	128.4 (2.9)	128.3 (1.3)	128.2 (2.2)
Calcium	mg	630	639 (70)	681 (58)	710 (56)
Phosphorus	mg	450	483 (27)	476 (23)	480 (30)
Magnesium	mg	58			
Chloride	mg	560			
Iron	mg	12			
Copper	mg	0.5			
Iodine	mg	0.1			
Zinc	mg	1.8			
Vitamin A	IU	2500	3056 (104)	3031 (49)	2921 (110)
Vitamin D	IU	400			
Vitamin E	IU	10			
Vitamin C	mg	100	82 (13)	79 (14)	78 (10)
Vitamin B_1	mg	1	0.97 (0.08)	0.94 (0.07)	0.91 (0.03)
Vitamin B_2	mg	1.6	1.76 (0.11)	1.77 (0.07)	1.73 (0.12)
Vitamin B_6	mg	0.5	0.47 (0.07)	0.47 (0.06)	0.47 (0.06)
Vitamin B_{12}	μg	4			
Niacin	mg	9	5.7 (0.7)	5.6 (0.8)	5.5 (0.7)
Folic acid	μg	100			
Pantothenic acid	mg	5			
Biotin	μg	99			
Vitamin K_1	μg	95			
Choline chloride	mg	100			
Carrageenan[a]	mg		7 (16)	255 (35)	1220 (142)

[a] Although carrageenan was added to the two carrageenan-containing formulas at levels of 300 and 1500 mg per liter, respectively, this is not supported by the analytical data. The analytical method used was a modification of Graham[21] for carrageenan in infant formulas in the range 200 to 300 mg per liter. In order to determine levels of 1500 mg per liter the product was diluted with noncarrageenan-containing formula to approximate a carrageenan content of 300 mg per liter, but the validity of this technique is not known.

before the scheduled autopsy, the infant received water ad libitum but no formula. On the morning of the autopsy, the animal was anesthetized with an intramuscular injection of ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, N. Y.). Blood was drawn from a femoral vein for hematological and chemical analyses. The prosector opened the abdomen, immediately removed the alimentary tract in a continuous strip from the rectum to the midpoint of the esophagus, and opened the intestine, which was immersed in 10% buffered formalin, along the mesenteric attachment. He then pinned the alimentary tract, serosa down, to chipboard in fresh 10% formalin. At least two (H. C. McG. and H. S. W.) and sometimes a third observer (H. S.) examined the gut minutely as fresh specimens and after formalin fixation, usually with the aid of a hand lens and often with a dissecting microscope. One observer (H. S.) studied all fixed specimens of intestines at the completion of the study with the aid of a dissecting microscope. Fourteen blocks for histological sections were taken from standard sites in the alimentary tract, including seven from the colon.

Standard blocks from all major viscera, muscle, lymph nodes, and central nervous system were fixed in 10% formalin, embedded in Paraplast (Sherwood Medical Industries, Inc., St. Louis, Mo.), sectioned at 5 μ, and stained with hematoxylin and eosin. One observer (H. C. McG.) examined all slides microscopically, identified only with the animal's accession number. Another observer (H. S.) examined all sections of the alimentary tract. Selected specimens were stained with the Prussian blue reaction and the periodic acid-Schiff reaction.

Microscopic measurements were performed with the aid of a calibrated eyepiece micrometer (Zeiss) using the procedure outlined by Needham and George.[23]

Statistical methods. Statistical methods for each set of data are described with the results. Because diet treatments were quantitative levels of a factor (amount of carrageenan per liter), we tested diet effects with contrasts for linear and quadratic trends.[24, 25] Sex was examined as a factor in the responses only with regard to weight.

Results

Formula consumption. The total formula consumed by each animal from birth through 105 days was analyzed by analysis of variance (ANOVA). A statistically significant ($P \approx 0.05$) linear trend was associated with carrageenan content. The means were 35,949 g for the no carrageenan group, 34,252 g for the 300 mg per liter group, and 38,899 g for the 1500 mg per liter group. The estimated pooled within group variance was 16,009,656. The finding of a linear trend by statistical test in contrast with a quadratic relationship apparent from inspection of the mean values is explained by the large variance. We interpreted the small differences between means as indicating that the formula group differences are not significant biologically.

Growth. We analyzed weights at 9-day intervals for the first 15 weeks with a multivariate model.[26] No linear or quadratic trends associated with carrageenan content of formula, no sex effect, and no diet by sex interaction were detected. Means for diet-sex treatment groups by age are presented in figure 1.

Organ system weights at autopsy. We compared organ weights by analysis of covariance[27] using body weight at autopsy as the concomitant variable. No statistically significant linear or quadratic trends were associated with carrageenan content of the formula.

Urine and feces. Daily gross observations of the color and estimated volume of the urine and of the color, amount, consistency, and other abnormalities of the feces showed no differences among the three formula groups. Twelve of a total of 333 tests on stools for occult blood were positive, all in the first 8 weeks. Positive tests for occult blood were not associated with carrageenan content of the formula.

Physical examination. Occasional minor physical abnormalities, unrelated to the carrageenan content of the formulas, were found in the 1st and 4th week examinations. Except for these, the physical examinations were normal.

Hematologic variables. The hemoglobin and hematocrit values were slightly higher and the white blood cell values slightly lower than those reported for maternally reared infant baboons from the SFRE colony.[28] There was no systematic pattern of variation of hematologic values after 1 month. Because the means for the formula groups at birth were quite different, the 1- through 4-month values were analyzed by analysis of covariance,[24] using the initial value as a concomitant variable. No statistically significant trends in hematologic determinations were detected.

Blood chemical analyses. The data were analyzed by ANOVA.[27] There was a statistically significant ($P < 0.05$) linear trend for sodium, but the difference did not appear significant biologically. The means for sodium were 146.3, 146.9, and 149.5 mEq per liter for the three diet groups with estimated variance of 6.68.

Alimentary tract. Although some abnormalities were observed in the alimentary tract as noted below, none was associated with carrageenan content of the formulas. No gross or microscopic lesions were observed in the tongue, mouth, esophagus, stomach, duodenum, je-

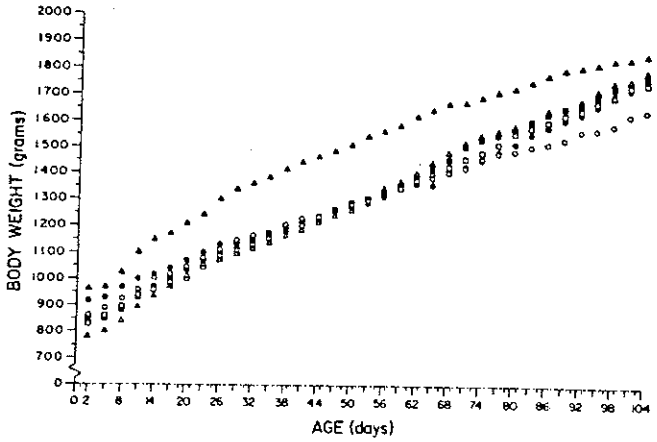

FIG. 1. Mean weights for diet-sex groups at 3-day intervals. Groups are represented as:

Carrageenan	Male	Female
mg/liter		
None	■	□
300	●	○
1500	▲	△

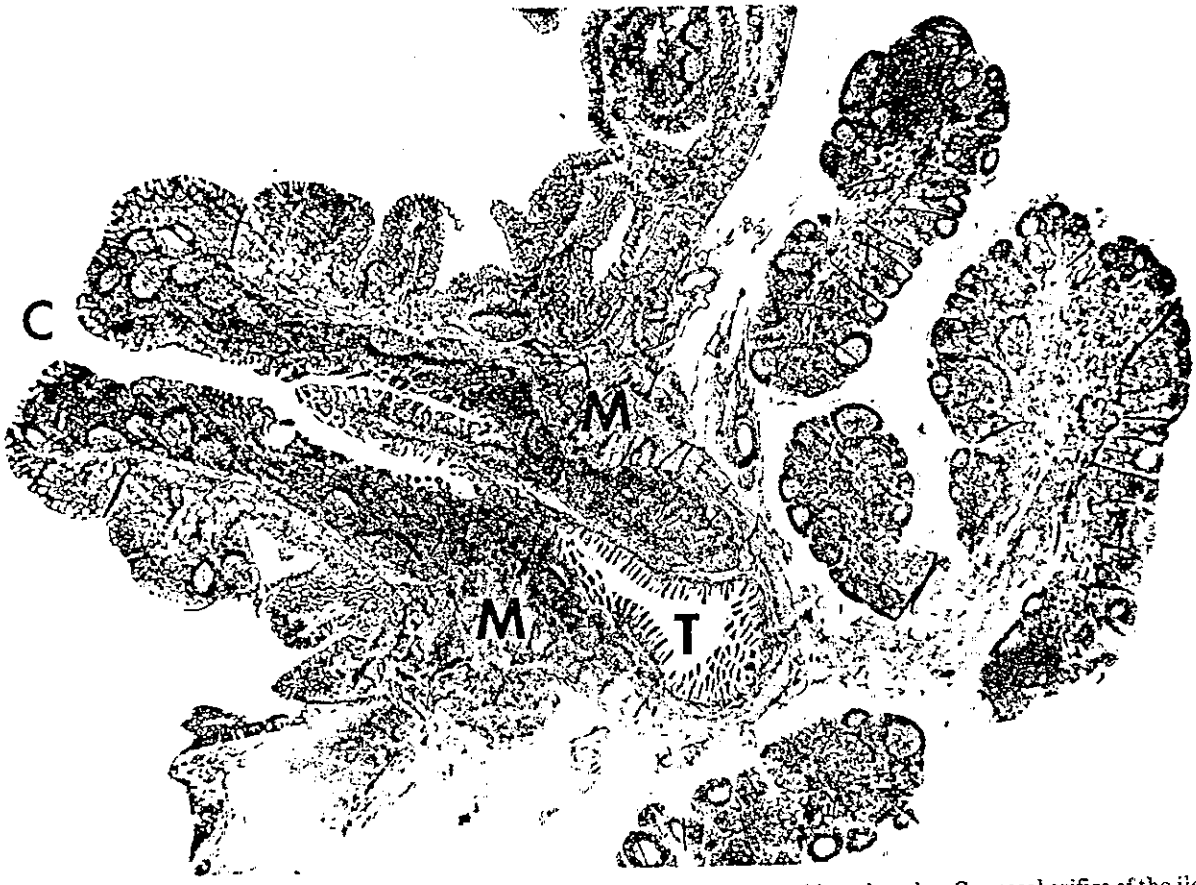

FIG 2.—Ileocecal valve; frontal section of the ileoceco-colic junction and adjacent ileocecal lymph nodes. *C* = cecal orifice of the ileal papilla. *T* = terminal ileum. *M* = ileal sphincter muscles. Note the abundance of lymphatic tissue in the mucosa and submucosa. Compare the height of the ileal villi with the diameter of the germinal centers which stand out as lighter gray, round and ovoid regions from the darker gray, diffusely distributed, gut-associated lymphatic tissue. The draining lymph nodes show reactive hyperplasia (H & E stain; original magnification ×3)

junum, or ileum. Microscopically, lymph follicles in the small intestine were frequent and had reactive germinal centers.

The baboon has no appendix. The ileocecal valve, which is formed by a protrusion of the ileal mucosa into the lumen of the cecum, is rich in lymphatic tissue. This structure measured approximately 5 mm in diameter and 5 to 10 mm in length, and a typical longitudinal section is shown in figure 2.

In most animals, the colonic surface of the ileocecal valve was reddened up to 75% of the circumference of the orifice. The discoloration did not extend cephalad to the mucosa lining the channel in the ileocecal valve, did not extend beyond the base of the valve on the colonic surface, and did not involve ulceration of the mucosal surface.

We evaluated the reddening of the ileocecal valve by reviewing gross photographs and the autopsy protocols without knowledge of formula group. No reddening was graded as 0; up to 25% of the circumference involved was graded as +; and more than 25% was graded as ++ (table 2). There was no apparent association of the reddening of the ileocecal valve with the carrageenan content of the formula. Independent grading of the photographs by two authors (H. C. McG. and H. S. W.) agreed on 20 of the 24 specimens (0.83). The kappa statistic[29] was 0.55 ($P < 0.001$, one-tailed test), indicating significant agreement beyond that expected by chance. Agreement within observer was of the same order as agreement between observer.

The mucosa of the cecum and of the remaining colon also frequently contained fine red streaks from 2 to 10 mm in length, oriented at right angles to the long axis of the colon.

Microscopic examination of the colon in the reddened areas showed only marked dilatation and engorgement of capillaries and venules just beneath the epithelium and to a lesser degree deeper in the lamina propria. In a few instances, there were deposits of granular brown pigment beneath the dilated vessels. This pigment gave a positive reaction for iron in a Prussian blue reaction and was presumed to represent hemosiderin.

No unequivocal ulcer was found in the colon on gross examination with the unaided eye or with a dissecting microscope. A few areas that appeared suspicious were not confirmed microscopically. Infrequent microulcers and cryt abscesses were noted in the standard microscopic sections from several animals, and spirochetosis

TABLE 2. *Presence and severity of selected colonic features by infant baboon and form a group*

Carrageenan in formula	Animal no	Hyperemia of ileocecal valve	Hyperemia of colon mucosa	Spirochetosis	Microulcers	Crypt abscesses
mg/liter						
None	X-46	++	+	+	0	0
	X-48	+	+	0	0	+
	X-53	++	+	+	+	0
	X-56	++	+	0	0	0
	X-62	+	0	+	0	0
	X-77	++	0	0	0	0
	X-79	++	+	0	0	0
	X-132	++	0	0	0	0
Total		8	5	3	1	1
300	X-42	++	+	+	+	+
	X-43	++	+	0	0	0
	X-44	++	+	0	0	+
	X-57	++	+	+	0	0
	X-72	0	0	0	0	0
	K-75	++	+	0	0	+
	X-78	++	0	0	0	0
	X-80	++	+	0	0	0
Total		7	6	2	1	3
1500	X-22	++	+	+	0	0
	X-45	++	+	0	0	0
	X-54	++	+	+	0	0
	X-61	++	+	+	0	+
	X-64	++	+	0	0	+
	X-66	++	+	0	0	0
	X-73	+	0	0	0	+
	X-81	++	+	0	0	0
Total		8	7	3	0	3

was present in some animals, but no lesion was associated with carrageenan content of the formula. Independence of diet groups and presence or absence of the various lesions given in table 2 was tested by a χ^2 test.[30] In no case was there a statistically significant association.

Next to the ileocecal valve, the gut-associated lymphatic tissue was best developed in the colon. Lymph follicles were frequent, had highly active germinal centers, were commonly aggregated, and measured 1160 by 675 μ in a typical example. The thickest colonic mucosa was in an animal of the no-carrageenan group where it measured 290 μ. The lamina propria was packed with lymphocytes, macrophages, some plasma cells, and occasionally a sprinkling of polymorphonuclear leukocytes, particularly in animals with crypt abscesses.

Incidental findings at autopsy. The incidental findings at autopsy were minor and unrelated to the presence or absence of carrageenan in the diet.

Discussion

Lack of effect of carrageenan. The primary objective of this study was to determine whether native undegraded carrageenan in the dietary formula produced any deleterious effects on the infant. Comparison of data on the animals in this experiment with the limited data on artificially or naturally reared infant baboons[31] indicate that these infants developed normally, and that native carrageenan has no influence on formula consumption, weight, gross characteristics of urine or feces, hematologic variables, blood chemical variables, organ weights, or gross and microscopic tissue characteristics of the infant baboon, even when given in five times the concentration customarily supplied in commercially available human infant formulas. This conclusion applies only to native or undegraded carrageenan as used in infant formulas.

Significance of occult blood in feces. The fecal samples containing occult blood were not different in color or consistency. Fecal occult blood has been recognized as a manifestation of intestinal allergy in human infants,[32–36] but the infant baboons showed no evidence of immune tissue damage as seen in human milk allergy.[37] Furthermore, they showed no anemia, hypoalbuminemia, or other signs and symptoms of milk allergy as described by the authors cited above and others.[38–40] Occult blood in the stool may have been derived from leakage of hyperplastic and hyperemic vessels supplying the hyperplastic gut-associated lymphatic tissue. Whatever its cause or origin, it was not associated with carrageenan content of the formula.

Lymphatic hyperplasia and inflammatory lesions. Lymph follicles of the size and activity of those in the alimentary tract of these animals would be considered hyperplastic in human pathology. This feature was not associated either with the presence or concentration of carrageenan in the formula or with intestinal spirochetosis. Experience with rhesus monkeys and man sug-

gests that colonization with spirochetes is a symbiotic relationship which does not elicit a recognizable inflammatory or immunologic tissue response.[41]

Both the hyperplasia of lymphatic tissue and the inflammatory lesions of the colon were present in all three groups of animals, regardless of the presence or concentration of carrageenan in the formula, and were not accompanied by deficiencies in growth or abnormalities in hematologic or biochemical variables. These findings are attributable, perhaps, to antigenic stimulation by exogenous milk proteins.

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