ESSENTIAL FATTY ACID STATUS AND CHARACTERISTICS ASSOCIATED WITH COLOSTRUM-DEPRIVED GNOTOBIOTIC AND CONVENTIONAL LAMBS. GROWTH, ORGAN DEVELOPMENT, CELL MEMBRANE INTEGRITY AND FACTORS ASSOCIATED WITH LOWER BOWEL FUNCTION[1]

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Summary

A factorial experiment involving gnotobiotic (GN) and conventional (CV) colostrum-deprived lambs and diets formulated to be adequate or deficient in linoleic acid was conducted to determine the effect(s) of the intestinal microflora on the essential fatty acid (EFA) status of the host and subsequent physiological consequences, i.e., growth, organ development, cell membrane integrity and lower bowel function. Lambs were obtained by sterile surgical procedures and housed in sterile isolators or in conventional metabolism stalls for 60 d. Skimmed cow's milk with 6% hydrogenated coconut oil and vitamins A, D and E added with and without .32% of the total calories as linoleic acid was homogenized, bottled and autoclaved, then fed to appetite three to four times daily. The GN lambs supplemented with linoleic acid gained significantly faster between 13 and 41 d of age and more efficiently between 27 and 41 d than the other treatment groups. The absence of dietary linoleic acid decreased liver and spleen weights and, in general, suppressed development of organs except the brain. Red blood cell hemolysis was not affected by treatment. Although showing signs of chronic mild diarrhea, the GN neonatal ruminant differed in Cl^- concentration and dry matter percentage of its lower bowel contents from the "classic rodent model." The results indicate that neonatal colostrum-deprived lambs have an EFA requirement, as evidenced by decreased growth and performance characteristics in the GN linoleic deficient vs GN supplemented group, and suggests that the required level is in excess of .32% of the total caloric intake as linoleic acid. Furthermore, the required level of this EFA may be elevated in the presence of the host microflora.

(Key Words: Gnotobiotic Lambs, Essential Fatty Acids, Linoleic, Growth.)

Introduction

The essential fatty acid (EFA) status of ruminants has been less extensively researched than that of nonruminants (Noble and Shand, 1982). Gullickson et al. (1942) reported that calves fed a low fat diet did not develop EFA deficiency symptoms, but growth was suppressed. Lambert et al. (1954) and Cunningham and Loosli (1954) found that calves raised on fat-free diets exhibited poor growth vs fat supplemented controls. Sklan et al. (1972) found correlated increases in plasma EFA of calves fed linoleic acid as .01, .32 or 1.00% of the total calories. Leat (1966) and Noble et al. (1971, 1972) also noted increases in plasma 18:2n6 levels of nursing lambs, suggesting that colostrum provides EFA. However, EFA in colostrum is <1% of the total fatty acids (Noble et al., 1971, Noble and Shand, 1982) and this implies that if the newborn

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ruminant requires EFA, it is capable of better EFA utilization and(or) its requirement is less than that of nonruminants. While there is an apparent biochemical EFA deficiency in neonatal lambs, it is alleviated after 10 d of nursing (Noble et al., 1972); the induction of EFA deficiency in adult ruminants cannot be readily demonstrated (Palmquist et al., 1977). Therefore, to study the neonatal ruminant's EFA status and the influence of the intestinal microflora on linoleic acid utilization, lambs maintained either gnotobiotic or conventional, were fed diets deficient or supplemented with linoleic acid, and the following variables were assessed: 1) performance, 2) feed utilization, 3) organ development, 4) membrane fragility and 5) electrolyte composition of lower bowel contents.

Materials and Methods

Crossbred neonatal lambs (¾ Suffolk and ¼ Rambouillet) were delivered either by Caesarean section or by hysterotomy. The Caesarean-derived group reached parturition in early spring and the hysterotomy-delivered group were procured in the fall of the same year. Ewes were brought to estrus, mated and conception dates were recorded. The lambs were all delivered 3 to 4 d prior to expected parturition. A total of 28 lambs were delivered and assigned randomly to either gnotobiotic (GN, known flora) conditions (12 animals) or maintained conventionally (CV, maintained in a clean, but not sterile environment; 16 animals). The gnotobiotic lambs were housed in custom-built sterile flexible film isolators (Trexler, 1971). Surgical supplies, food and maintenance equipment were introduced via a portable transfer cylinder through double lock entry ports after sterilization with peracetic acid, ethylene oxide, or by autoclaving.

Diets were fed immediately after delivery to the neonatal lambs to minimize the effect of colostrum on "storing EFA." Hydrogenated

coconut oil (HCO) was included in the diet to serve as a vehicle for fat soluble vitamins and to provide a high caloric density. The diet was formulated including skimmed milk prepared by hot process extraction containing <.01% total fat[6], with 6% (weight) HCO added to the skim milk diet. Hydrogenated coconut oil fatty acids and wt percentage were respectively: 8:0, 10.7; 10:0, 7.2; 12:0, 50.0; 14:0, 16.6; 16:0, 8.1; 18:0, 8.3. Supplemental vitamins dissolved in the HCO included: E − (DL tocopherol, 1.0 IU/g linoleic acid)[7]; A − (vitamin A palmitate, 1,800 IU/kg diet)[7] and D − (calciferol, 110 USP/kg diet)[8]. Linoleic acid (99% pure) was added to the diet (EFA supplemented) as .32% of the total calories (Cunningham and Loosli, 1954). These ingredients were mixed with or without the linoleic acid added in a stirring batch pastuerizer[9] (10 C, 20 min), homogenized[10] using 50 and 200 kg/cm^2 pressure, respectively, and bottled in 2-liter flasks[11]. Prior testing with Bacillus stearothermophilus capsules and spore strips[12] showed that autoclaving at 121 C at 1 kg/cm^2 for 25 min produced a reliably sterile product. The autoclave used was equipped with pulsating pressure and automatic timers. Bottles were cooled in a walk in refrigeration unit to 5 C and then removed and stored at 20 C until they were introduced into the isolator. Prior to placement into the isolator, the bottles were washed with a mild soap and chlorox solution and then surface sterilized in the entry port with 2% peracetic acid spray (minimum 30 min exposure).

The colostrum-deprived GN and CV lambs were fed the sterile milk solution (with or without linoleic acid) initially four times a day at approximately 6-h intervals (24 h/d, 7 d/wk) and later, three times daily. The lambs were fed to satiety during this time and consumption was recorded daily. Lambs were weighed weekly. Germfree status of the animals was verified as described by Wagner (1959). Jugular blood was sampled weekly from GN and CV lambs (.3 ml sodium heparin, 1,000 U/ml)[13]. After centrifugation, plasma samples were stored at −20 C under N$_2$ and the packed red blood cells were used immediately for testing hemolysis. Red blood cell fragility (RBCF) was measured as hemolysis using osmotic gradient NaCl solutions (Winthrobe, 1947). At 2 mo of age, the GN lambs were removed from the isolator and terminated by a cervical blow followed by carotid exsanguination. The organs were removed, weighed and

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TABLE 1. AVERAGE DAILY GAINS AND FEED EFFICIENCY FOR GNOTOBIOTIC (GN)
AND CONVENTIONAL (CV) LAMBS FED DIETS WITH (L)
OR WITHOUT (D) LINOLEIC ACID

Treatment	No.	13 to 27 d after birth		27 to 41 d after birth	
		gain, g/d[a]	gain/milk[a]	gain, g/d[a]	gain/milk[a]
GNL	4	209 ± 18[b]	.198 ± .016[b]	131 ± 30[b]	.115 ± .011[b]
GND	3	91 ± 18[c]	.249 ± .052[b]	41 ± 5[c]	.065 ± .009[c]
CVL	4	82 ± 41[cd]	.096 ± .048[c]	41 ± 27[c]	.052 ± .028[c]
CVD	3	62 ± 2[e]	.078 ± .019[c]	32 ± 14[c]	.033 ± .030[c]

[a]Mean ± SE.

[b,c,d]Values within columns bearing different superscripts differ (P<.05).

preserved in .9% NaCl at −20 C until further analysis. Digesta were collected from the small intestine, cecum and colon and stored at −20 C.

Lipids in plasma and liver samples were extracted (Bligh and Dyer, 1959), saponified, methylated with boron trifluoride at 14% in methanol and the methyl esters were quantified by GLC[14] using known reference standards[15] (Bruckner et al., 1982). A glass column 183 cm packed with 10% EGSSX — gas Chrom Q 100/200 mesh was used; nitrogen flow rate was 40 ml/min with a 2 C/min temperature program (165 to 205 C).

Dry matter of intestinal contents from each of the lambs was determined in triplicate after drying in a vacuum oven for 24 h at 60 C. Osmolality (mOsm/liter) of the intestinal content supernatant (20,000 × g for 20 min) was determined using freezing point depression[16]. Relative viscosity of the intestinal supernatants was measured at 37 C using an Oswald viscometer tube[17]. The lower bowel content supernatants were assayed for Na^+ and K^+ using a flame photometer[18] and for Cl^- by the coulometricamperometric titration technique with silver ions[19].

Statistical Analysis. Comparisons were made using two-way analysis of variance and Student's t-test to indicate possible flora and nutrient interactions (Snedecor and Cochran, 1967).

Results and Discussion

General. The survival rate of the GN lambs was 75% (two of 12 died due to intestinal strangulation and one died due to respiratory failure). Of the nine remaining GN lambs, one deficient and one supplemented with linoleic acid became associated with a micrococus species[20]. Hydrogenation of linoleic acid could not be demonstrated for this organism. The isolator-maintained group was therefore designated as GN.

Survival was a greater problem for CV colostrum-deprived than GN lambs, and survival of the linoleic acid-deficient CV animals was extremely jeopardized. Four of the seven linoleic acid supplemental CV animals survived the 2-mo trial period, while only three of the nine deficient CV lambs survived for the 2-mo period.

Fecal microbial monitoring of CV lambs for 2 wk after birth indicated that prior to death, the fecal counts of enterobactericae were elevated, [i.e., increased from 10^7 to 10^9 colony-forming units (CFU)/g feces]. Feces of colostrum-deprived CV lambs were devoid of detectable lactobacillus organisms regardless of linoleic acid status. Lambs of comparable age, not included in this study, which were normally delivered by the ewe and subsequently nursed showed lactobacillus counts as high as 10^9 CFU/g feces. It was apparent that the colostrum-deprived CV lambs were greatly penalized in

[14]Varian 2100, Melbourne, Australia.

[15]Nu Chek-Prep Elysian, MN. The methyl 5, 8, 11 eicosatrienoic acid was provided by Dr. Howard Sprecher, Columbus, OH.

[16]Advanced Instruments Osmometer, Inc., Decatur, MA.

[17]Thomas Scientific Apparatus, Philadelphia, PA.

[18]Instrumentation Lab., Inc., Lexington, MA.

[19]Buchler-Cotlove Chloridometer, Buchler Instruments, Inc. Fort Lee, NJ.

[20]Micrococcus pyogenes var. albus, elucidated through the kind cooperation of Steve Jackson, Dept. of Microbiology, Univ. of Kentucky, Lexington.

survival rates by the microbial stress imposed upon them vs their isolator counterparts. After the initial trauma of exposure, the performance of the CV lambs improved, but never equalled the performance of the GNL lambs.

Performance. As shown in table 1, average daily gains (ADG) of the supplemented GN group were greater (P<.05) than those of the other treatment groups. The comparison between GN-supplemented vs GN-deficient indicates most directly that the lack of linoleic acid in the diet adversely affects the performance of the neonatal colostrum-deprived lambs. Comparisons of feed efficiencies (table 1) indicate that shortly after birth, the CV animals, regardless of linoleic acid status, had lower (P<.05) feed efficiencies than the GN counterparts. During the latter phase of treatment, this difference was less evident. Small sample size, and the variability encountered, make conclusive interpretations difficult: however, it was evident that the performance of the GN-supplemented lambs was superior to all other treatment groups, indicating that both linoleic acid status and microbial status of the neonatal ruminant are critical to performance. The colostrum-deprived CV lamb, irrespective of linoleic acid status, did not perform as well as lambs isolated in the protected environment. The presence of the microflora and the lack of linoleic acid in the diet seem to place an additive stress on neonatal lambs. These performance observations confirm the results of others (Gullickson et al., 1942) with young EFA-deficient ruminants and indicate that the presence of the microflora placed additional stress on the performance of the neonatal colostrum-deprived lambs.

Organ Development. The organs of the body develop at different rates, and this development may be influenced by a variety of factors. Brain weights of young growing lambs do not reflect the effects of a low plane of nutrition as markedly as liver or spleen weights (Palsson and Verges, 1952). Linoleic acid deficiency resulted in lower (P<.05) liver and spleen weights (table 2). Although not differing significantly, most of the other organs were smaller in the deficient groups. The organ weights were in the following order: GN-supplemented > CV-supplemented > GN-deficient > CV-deficient, suggesting a correlation with EFA status. The brain did not follow this pattern. The combined effects of a linoleic acid deficiency and the presence of the microflora may retard heart and lung develop-

TABLE 2. MEAN BODY WEIGHTS (BWT.KG) AND ORGAN WEIGHTS (G) OF GNOTOBIOTIC OR CONVENTIONAL LAMBS FED DIETS WITH (L) OR WITHOUT (D) LINOLEIC ACID

Treatment	No. of animals	BWT	Liver	Spleen	Brain	Heart	Lungs	Kidneys	Adrenals	Abomasum	Omasum	Rumen-reticulum	Cecum	Large intestine	Small intestine
GNL	4	10.7a	267.7a	19.9a	63.6	67.5	151.4	58.3	1.5	80.1	19.1a	50.3a	47.0	98.1	273.0a
GND	3	5.6b	127.1b	8.8b	63.7	50.7	119.4	42.8	1.4	40.4	10.7b	29.2b	33.3	56.9	141.7b
CVL	4	5.7b	215.0ab	20.3a	64.5	56.6	159.8	47.9	1.9	46.3	11.9b	27.5b	27.4	63.8	196.7ab
CVD	3	5.7b	139.2ba	11.1b	65.3	38.2	98.7	32.0	1.3	37.0	8.2b	23.1b	24.7	48.2	177.3ab
EMSc		4.1	5,712.4	20.6	25.9	310.9	2,750	213.2	.2	755.6	9.3	102.1	208.6	868.9	4,673.9

[a,b] Values within columns bearing different superscripts differ (P<.05).

[c] Error mean square.

TABLE 3. MEAN EICOSATRIENOIC TO EICOSATETRAENOIC FATTY ACID RATIO
IN PLASMA AND LIVER OF GNOTOBIOTIC (GN) AND CONVENTIONAL (CV)
LAMBS FED DIETS WITH (L) OR WITHOUT (D) LINOLEIC ACID

| | | Tri:tetra[a] | |
Treatment	No.	Plasma	Liver
GNL	4	.7[bc]	.6[b]
GND	3	2.0[bd]	2.3[c]
CVL	4	.8[c]	.7[b]
CVD	3	2.1[d]	1.7[c]
EMS[e]		.5	1.2

[a] 20:3n9: 20:4n6 ratios — based on weight percentage of total plasma and liver fatty acids.

[b,c,d] Values within column bearing different superscripts differ (P<.05).

[e] Error mean square.

ment as indicated by the CV-deficient vs CV-supplemented group; however, this difference was not significant. The heavier omasum, abomasum and rumen-reticulum weights of CN-supplemented lambs in comparison with other treatment groups suggest that the development of these organs paralleled body weight gains. The body weights of the GN-deficient, CV-supplemented and CV-deficient groups were similar as were the weights of the aforementioned organs. The presence of the microflora influenced organ development, with lower organ weights for CV-supplemented vs GN-supplemented lambs. These data suggest that a dietary deficiency of linoleic acid affects organ weights of neonatal lambs somewhat similar to low planes of nutrition (Palsson and Verges, 1954).

Hematologic Factors. There were no significant differences observed in hematocrit except a general decrease with increasing age from ~45 to ~35%. No differences in RBCF were observed in this experiment. While perhaps due to differences between ruminant and nonruminant red blood cell lipoprotein composition, it is more likely that all of the lambs were slightly EFA deficient (indicated by plasma and liver triene:tetraene fatty acid ratios above .4; table 3) which masked differences due to status.

Intestinal Content Composition. The GN animals, in general, exhibited mild chronic diarrhea. This condition might be causally related to the low chloride levels and elevated mucopolysaccharide content found in the lower bowels of GN animals (Gordon and Wostmann, 1973). The cecal contents of the GN-supplemented lambs (table 4) exhibit characteristics

that have also been noted for germ-free (GF) rodents, i.e., decreased Cl content and increased relative viscosity, in comparison with the CV counterparts (Bruckner and Szabo, 1984). However, the cecal dry matter content was similar for supplemented GN and CV lambs, which differs from the decreased dry matter content observed for the GF vs CV rodents (Gordon and Pesti, 1971). Also, the concentration of Na in cecal contents was lower for supplemented GN than supplemented CV lambs, a response not noted for GF vs CV rodents (Asano, 1967). The dry matter content of the CV-supplemented colon samples shows a marked increase from that observed for the cecum. This increase is absent in the GN-supplemented colon samples and is indicative of the observed chronic mild diarrhea (indicated by the decreased dry matter and osmolarity of the colon contents), this anomaly was not analogous to the chronic diarrhea found in GF rodents. Although the supplemented GN had a decreased Cl level in the cecum, the amount present did not seem to constitute a deficiency capable of creating a water imbalance. No differences were noted in the cecal dry matter between supplemented GN and CV. Another deviation from the rodent cecal situation is the lower Na levels observed in the supplemented GN cecum. These observed differences suggest an apparent deviation from the mechanism(s) currently cited for explaining the germ-free rodent diarrhea status in comparison with those for the GN-supplemented lambs. In rodents, an increase in the relative viscosity of intestinal contents has been associated with parallel increases in mucopolysaccharides (Gordon and

TABLE 4. MEAN INTESTINAL CONTENT CHARACTERISTICS OF GNOTOBIOTIC (GN) AND CONVENTIONAL (CV) LAMBS

Item	No.	Dry matter, %	Osmolarity, mOsm/liter	Relative viscosity, viscosity of material/ viscosity of H_2O	Na	K	Cl
					meq/liter		
GN[a]							
Small intestine	3	6.7	288	1.7	91	22	97
Cecum	3	19.4	251	3.1[b]	29[b]	21	36[b]
Colon	4	20.2[b]	506[b]		137	33	152
CV[a]							
		EMS[d]	EMS	EMS	EMS	EMS	EMS
Small intestine	3	4.7 / 1.6	396 / 4,604	1.7 / .04	77 / 2,256	28 / 65	61 / 640
Cecum	3	17.1 / 78.0	310 / 23,078	1.7[c] / .36	112[c] / 559	28 / 230	71[c] / 414
Colon	3	43.1[c] / 53.7	993[c] / 46,056		193 / 1,429	67 / 1,997	141 / 162

[a]Only animals supplemented with linoleic acid were used for comparison.

[b,c]Values within columns for similar segments bearing different superscripts differ (P<.05).

[d]Error mean square.

Wostmann, 1973). The increased viscosity of the GN-supplemented cecal contents might suggest a different type or amount of mucin secretion. If this mucin is undegraded by the microflora (as would be the case in GN-supplemented), it might contribute to the increased water retention observed in the GN-supplemented colon contents.

General Discussion

Newborn lambs exhibit biochemical fatty acid characteristics, triene:tetraene ratios above .4, which are indicative of an EFA deficiency condition (Noble et al., 1971; Noble, 1981). In the present study, 2-mo-old colostrum-deprived lambs raised either: (1) in the presence or absence of a microflora, and (2) fed diets with or without .32% of the calories as linoleic acid, exhibited a variety of interactive performance characteristics. Lambs that did not receive dietary EFA (GND and CVD) gained weight slowly and had rough hair coats, which are symptoms associated with an EFA deficiency. Furthermore, conventional lambs supplemented with linoleic acid performed similarly to GN and CV deficient animals, indicating that not only the presence of EFA, but alo the presence of the microflora influence the well being of the animal. A detrimental additive effect of nutritional and microbial stress is suggested by the increased mortality of the CV-deficient vs CV-supplemented lambs. Based on growth rates and feed efficiency, it would appear that linoleic acid at .32 energy percentage is adequate for the performance of lambs in the absence of microbial stress, i.e., GN-supplemented. However, in the presence of the microflora, lamb performance becomes jeopardized at .32% linoleic acid. Furthermore, the plasma and liver triene:tetraene fatty acid ratios suggest that even the GN-supplemented lambs have a slight EFA deficiency. In the absence of dietary linoleic acid, the liver and the spleen weights were drastically reduced, suggesting that the EFA levels are more critical for these organs than other organs, and may more accurately reflect alteration in EFA status. Characteristics of lower bowel content indicate that while the GN-supplemented vs CV-supplemented lambs exhibit mild chronic diarrhea (decreased colon dry matter content), the mechanisms responsible for this observed anomaly seem to differ from the classic rodent model. Based on performance and triene:tetraene ratios, neonatal colostrum-deprived lambs have an EFA requirement that

is in excess of .32% of energy. Furthermore, the required level of EFA may be elevated in the presence of the host microflora.

Literature Cited

Asano, T. 1967. Inorganic ions in cecal content of gnotobiotic rats. Proc. Soc. Exp. Biol. Med. 124:424.

Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911.

Bruckner, G., J. Shimp, S. Goswami, J. Mai and J. E. Kinsella. 1982. Dietary trilinoelaidate: Effects on metabolic parameters related to EFA metabolism in rats. J. Nutr. 112:126.

Bruckner, G. and J. Szabo. 1984. Nutrient absorption in gnotobiotic animals. In: H. Draper (Ed.) Advances in Nutrition Research, Vol. 6. (In press).

Cunningham, H. M. and. J. K. Loosli. 1954. The effect of fat free diets on young dairy calves with observations on metabolic fecal fat and digestion coefficients for lard and hydrogenated coconut oil. J. Dairy Sci. 37:453.

Gordon, H. A. and L. Pesti. 1971. The gnotobiotic animal as a tool in host microbial relationships. Bacteriol. Rev. 35:390.

Gordon, H. A. and B. S. Wostmann. 1973. Germfree Research. J. B. Henaghan (Ed.) Academic Press, New York. p 593.

Gullickson, T. W., F. C. Fountaine and J. B. Fitch. 1942. Various oils and fats as substitutes for butterfat in the ration of young calves. J. Dairy Sci. 25:117.

Lambert, M. R., N. L. Jacobsen, R. S. Allen and J. H. Zaletel. 1954. Lipid deficiency in the calf. J. Nutr. 52:259.

Leat, W.M.F. 1966. Fatty acid composition of the plasma lipids of newborn and maternal ruminants. Biochem. J. 98:598.

Noble, R. C. 1981. Lipid metabolism in the neonatal ruminant. In: W. W. Christie (Ed.) Lipid Metabolism in Ruminant Animals. Pergamon Press, Elmsford, NY. p 411.

Noble; R. C. and J. H. Shand. 1982. Fatty acid metabolism in the neonatal ruminant. In: H. Draper (Ed.) Advances in Nutrition Research, Vol. 4. p 287.

Noble, R. C., W. Steele and J. H. Moore. 1971. Diet and the fatty acids in the plasma of lambs during the first eight days after birth. Lipids 6:26.

Noble, R. C., W. Steele and J. H. Moore. 1972. The metabolism of linoleic acid by the young lamb. Brit. J. Nutr. 27:503.

Palmquist, D. L., W. Mattos and R. L. Stone. 1977. Absence of eicosatrienoic acid in plasma of sheep parenterally infused with a high glucose fat-free solution. Lipids 12:235.

Palsson, H. and. J. B. Verges. 1952. Effects of the plane of nutrition on growth and development of carcass quality in lambs. J. Agr. Sci. (Camb.) 42:1.

Sklan, D., R. Volcani and P. Budowski. 1972. Effects of diets low in fat or essential fatty acids on the fatty acid composition of blood lipids in calves.

Brit. J. Nutr. 27:365.

Snedecor, G. W. and W. G. Cochran. 1967. Statistical Methods (6th Ed.). Iowa State Univ. Press, Ames. p 299.

Trexler, P. C. 1971. Microbial isolation of large animals. Vet. Rec. 88:15.

Wagner, M. 1959. Determination of germfree status. Ann. New York Acad. Sci. 78:89.

Winthrobe, M. 1947. Clinical Hematology. Lea and Febiger, Philadelphia, PA.