Conjugated Linoleic Acid–Enriched Butter Fat Alters Mammary Gland Morphogenesis and Reduces Cancer Risk in Rats[1]

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ABSTRACT Conjugated linoleic acid (CLA) is a potent cancer preventive agent in animal models. To date, all of the in vivo work with CLA has been done with a commercial free fatty acid preparation containing a mixture of *c*9,*t*11-, *t*10,*c*12- and *c*11,*t*13-isomers, although CLA in food is predominantly (80–90%) the *c*9,*t*11-isomer present in triacylglycerols. The objective of this study was to determine whether a high CLA butter fat has biological activities similar to those of the mixture of free fatty acid CLA isomers. The following four different endpoints were evaluated in rat mammary gland: *1*) digitized image analysis of epithelial mass in mammary whole mount; *2*) terminal end bud (TEB) density; *3*) proliferative activity of TEB cells as determined by proliferating cell nuclear antigen immunohistochemistry; and *4*) mammary cancer prevention bioassay in the methylnitrosourea model. It should be noted that TEB cells are the target cells for mammary chemical carcinogenesis. Feeding butter fat CLA to rats during the time of pubescent mammary gland development reduced mammary epithelial mass by 22%, decreased the size of the TEB population by 30%, suppressed the proliferation of TEB cells by 30% and inhibited mammary tumor yield by 53% ($P < 0.05$). Furthermore, all of the above variables responded with the same magnitude of change to both butter fat CLA and the mixture of CLA isomers at the level of CLA (0.8%) present in the diet. Interestingly, there appeared to be some selectivity in the uptake or incorporation of *c*9,*t*11-CLA over *t*10,*c*12-CLA in the tissues of rats given the mixture of CLA isomers. Rats consuming the CLA-enriched butter fat also consistently accumulated more total CLA in the mammary gland and other tissues (four- to sixfold increases) compared with those consuming free fatty acid CLA (threefold increases) at the same dietary level of intake. We hypothesize that the availability of vaccenic acid (*t*11–18:1) in butter fat may serve as the precursor for the endogenous synthesis of CLA via the Δ9-desaturase reaction. Further studies will be conducted to investigate other attributes of this novel dairy product. J. Nutr. 129: 2135–2142, 1999.

KEY WORDS: • *conjugated linoleic acid* • *butter fat* • *mammary gland morphogenesis* • *mammary cancer prevention* • *tissue CLA isomers* • *rats*

Conjugated linoleic acid (CLA)[3] is a term that refers to a collection of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. Milk and other dairy products are good sources of CLA (Parodi 1997) because of the biohydrogenation of dietary unsaturated fatty acids by rumen bacteria. CLA is an intermediate in the biohydrogenation of linoleic acid, and a portion of the CLA in milk fat arises from CLA that has escaped complete rumen biohydrogenation. The other portion of CLA in milk fat is synthesized in the tissues by Δ9-desaturase from *trans*-11 18:1, another intermediate in rumen biohydrogenation (Griinari and Bauman 1999). Recent studies have shown that the milk fat content of CLA can be markedly enhanced by several dietary manipulations, especially those involving dietary additions of plant oils, which are high in unsaturated fatty acids (Griinari and Bauman 1999, Kelly et al. 1998).

CLA is a potent cancer preventive agent (Ip et al. 1994). In animal models of chemical carcinogenesis, CLA has been shown to inhibit skin papillomas (Belury et al. 1996), forestomach neoplasia (Ha et al. 1990), mammary tumors (Ip et al. 1996) and colon aberrant crypt foci (Liew et al. 1995). Moreover, CLA is also effective in reducing the size and metastasis of transplanted human breast cancer cells and prostate cancer cells in SCID mice (Cesano et al. 1998, Visonneau et al. 1997). In the rat model, Ip et al. (1995) also demonstrated that when CLA feeding was limited to only the period of

[1] Supported by grants from the National Dairy Council, Rosemont, IL and Kraft Foods, Inc., Glenview, IL; grant CA 61763 from the National Cancer Institute, National Institutes of Health, Roswell Park Cancer Institute Core grant CA 16056 awarded by the National Cancer Institute; and grant AIBS 2423 from the Department of Defense. Support was also received from Northeast Dairy Foods Research Center and Cornell University Agricultural Experiment Station.

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[3] Abbreviations used: AUOD, arbitrary unit of optical density; CLA, conjugated linoleic acid; MNU, methylnitrosourea; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator–activated receptor; TEB, terminal end bud.

0022-3166/99 $3.00 © 1999 American Society for Nutritional Sciences.

Manuscript received 1 June 1999. Initial review completed 29 July 1999. Revision accepted 3 September 1999.

pubescent mammary gland development, it was able to confer a lasting protection against the subsequent induction of mammary tumors. An evaluation of the digitized image of the mammary tree in whole mounts suggested that there was diminished epithelial branching as a result of CLA treatment (Thompson et al. 1997). This change in morphogenesis could account in part for the reduced susceptibility to cancer induction because of a decrease in the population of terminal end buds (Banni et al. 1999), which are the target sites of mammary carcinogenesis.

To date, all of the in vivo work with CLA has been done with a commercial free fatty acid preparation containing a mixture of 8,10-, 9,11-, 10,12- and 11,13-isomers, although CLA in food is predominantly (80–90%) the 9,11-isomer present in triacylglycerols. To date, there has been no information on whether CLA delivered as a constituent of food has biological activities similar to those of the mixture of CLA isomers delivered as free fatty acids. This was one of the objectives; thus, we fed dairy cows in a manner that allowed the production of high CLA butter. In addition to feeding a group of rats the high CLA butter, the design included two other groups that were given either a mixture of CLA isomers (obtained from Nu-Chek-Prep, Elysian, MN, hence designated as Nu-Chek CLA for convenience) or a synthetic CLA preparation consisting predominantly of the 9,11-isomer (obtained from Matreya, Pleasant Gap, PA, designated as Matreya CLA); both preparations provide CLA in the free fatty acid form. A major goal of this investigation therefore was to address the question whether different CLA isomers have different biological activities. The prepubertal rat model alluded to above (Ip et al. 1995, Thompson et al. 1997) was employed for this research. The following endpoints were used to assess the activities of the different sources of CLA: *1*) image analysis of mammary gland development; *2*) terminal end bud (TEB) density; *3*) proliferative activity of TEB cells; and *4*) mammary cancer prevention bioassay. Measurements of CLA isomer incorporation into tissues were also made to provide insight regarding the bioavailability of individual isomers.

MATERIALS AND METHODS

Production and analysis of high CLA butter fat. Holstein cows were used to produce the milk fat that was used to make butter. Cows were located at the Cornell University Teaching and Research Center; this portion of the study was approved by the Cornell University Institutional Animal Care and Use Committee. Control cows (n = 10) were fed a total mixed diet composed of concentrates plus corn silage as the roughage source. Cows (n = 20) used to provide the high CLA butter were fed a similar diet with the addition of 5.3% sunflower oil (Kelly et al. 1998). After 1 wk of consuming the sunflower oil diet, milk samples were obtained and the CLA content of the milk fat was determined. There was substantial individual variation in CLA concentration as reported previously (Kelly et al. 1998). Cows (n = 9) with the highest concentrations of CLA continued to consume the diet for a second week, and their milk was collected to make the high CLA butter.

Raw milk was pasteurized by the high temperature–short time method (model #3919, Alfa-Laval Type-P13-RCF 1982, Kenosha, WI) at 175°F for 18 s, then separated into cream and skim milk. The cream was vat pasteurized at 162°F for 30 min and stored in the cooler for 24 h. It was then churned (Zane Butter Churn Model #A, General Dairy Equipment, Minneapolis, MN) for 30 min at 50°F until butter was the size of popcorn kernels; then the buttermilk was drained off. The butter was rinsed and washed with 4°C water, and the unsalted butter was transferred to 0.5-kg plastic containers and kept at −20°C until use.

Fatty acid methyl esters for butter fat analysis were prepared by the procedure of Christie (1982) and determined by gas chromatography with the use of a Hewlett-Packard GCD system (Palo Alto, CA) equipped with HP G107A GCD software for peak integration (Kelly et al. 1998). Control butter and high CLA butter contained 5.1 and 41.0 mg CLA/g of fat, respectively. The fatty acid composition of these two types of butter is shown in **Table 1**. Saturated fatty acids (4:0–18:0) constituted ~67.7% of the total in control butter fat, but only 48.1% in high CLA butter fat. A major difference was accounted for by palmitic acid, 28.6% vs. 17.8%. The concentration of oleic acid was quite similar, i.e., 18.5% in control butter fat vs. 16.0% in high CLA butter fat. However, there was much more *trans*-octadecenoic acid in the high CLA butter fat (25.0%) than in the control butter fat (3.8%); a major *trans*-isomer was vaccenic acid (*t*11-C18:1). Despite the differences in their fatty acid composition, we decided to use the two kinds of butter fat as is, without making any further adjustment to the fatty acid composition of the rat diet. The argument is that the most important fatty acid for modulation of mammary carcinogenesis in the rodent model is linoleic acid (Ip 1987 and Ip 1997), and the level of this fatty acid was equivalent in the two kinds of butter fat.

Experimental protocol for rat feeding studies. Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) at weaning. Upon arrival, they were divided equally into four dietary groups: *1*) control butter fat, which provided 0.1% CLA in the diet; *2*) high CLA butter fat, which provided 0.8% CLA in the diet; 3) Matreya CLA; and 4) Nu-Chek CLA. Matreya CLA and Nu-Chek CLA, purchased from their namesake vendors located in Pleasant Gap, PA, and Elysian, MN, respectively, were added as free fatty acids to the diet to match the total level of butter CLA present in the second group. **Table 2** summarizes the butter fat content of the different diets, the composition of the remaining ingredients, the total CLA level in each diet and the major CLA isomers in the various sources of CLA. It should be noted that the analysis of CLA isomers in the commercial CLA preparations was done by one of the authors (S.B.), and is not based on information provided by the vendors. All rats were fed these four different diets for 1 mo (i.e., from 23 to 55 d of age) to prepare them for the following

TABLE 1

Fatty acid composition of control butter fat and high conjugated linoleic acid (CLA) butter fat

Fatty acid		Control butter	High CLA butter
		g/100 g total fatty acids	
Butyric	4:0	4.2	5.4
Caproic	6.0	2.5	1.4
Caprylic	8.0	1.5	0.7
Capric	10:0	3.5	1.5
Lauric	12:0	4.0	1.7
Myristic	14:0	12.0	7.4
Myristoleic	14:1	1.2	0.9
Pentadecylic	15:0	1.1	0.6
Palmitic	16:0	28.6	17.8
Palmitoleic	16:1	1.4	1.7
Margaric	17:0	0.5	0.3
Stearic	18:0	9.8	11.3
Oleic	*c*9-18:1	18.5	16.0
trans-Octadecenoic[1]	*trans*-18:1	3.8	25.0
Linoleic	*c*9,*c*12-18:2	2.8	2.9
Conjugated linoleic[2]	CD-18:2	0.5	4.1
γ-Linolenic	18:3	0.4	0.2
Others		3.7	1.1
	Total	100.0	100.0

[1] This represents total trans-18:1, of which vaccenic acid (*t*11-18:1) accounted for ~24.8 and 48.7% in control butter and high CLA butter, respectively.

[2] CD denotes conjugated diene.

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TABLE 2

Supplementation of conjugated linoleic acid (CLA) from different sources in the dietary design

Dietary treatment[1]	Total CLA in diet	Group designation	Major CLA isomers[2]	
	g/100 g		*%*	
20% (g/100 g) control butter fat	0.1	Control	83.0	c9,t11
			6.1	t7,c9
20% high CLA butter fat	0.8	Butter CLA	92.0	c9,t11
			4.8	t7,c9
20% control butter fat + 0.7% Matreya CLA	0.8	Matreya CLA	81.0	c9,t11
			17.5	c9,c11
20% control butter fat + 0.7% Nu-Chek CLA	0.8	Nu-Chek CLA	17.6	c11,t13
			36.5	t10,c12
			25.3	c9,t11
			15.3	t8,c10

[1] The remaining ingredients in the diet consisted of 23.5% casein, 44.8% dextrose, 4.1% AIN-76 mineral mix, 1.2% AIN-76A vitamin mix, 5.9% alphacel, 0.3% methionine and 0.2% choline bitratrate.

[2] The butter fat also contained trace amounts (<1%) of cis/trans 8,10-, 10,12- and 11,13-CLA isomers. However, the remaining CLA isomers in Matreya CLA and Nu-Chek CLA are mainly c,c- or t,t-isomers.

four sets of experiments: *1*) digitized image analysis of mammary epithelium in whole mounts, $n = 9$; *2*) quantitation of TEB density in the mammary gland and assessment of proliferative activity in TEB cells, $n = 6$; *3*) mammary carcinogenesis bioassay, $n = 30$; and *4*) measurements of CLA isomers in tissues, $n = 6$.

Preparation of the mammary gland for image analysis. The abdominal-inguinal mammary gland chain was excised in one piece and stretched onto a 75 × 50 mm^2 microscope slide. The whole mount was fixed in methacarn for 12–18 h and rinsed in 70% ethanol. It was then dehydrated using a series of ethanol solutions (70, 95 and 100%) for 1 h each and cleared in xylene for 2 h. The tissue was rehydrated with descending grades of ethanol and immersed in fresh 0.4% alum carmine stain for 3 d. Once staining was completed, the whole mount was dehydrated using ethanol as described above and cleared with one change of xylene for 2 h. Each whole mount was then placed in a 10 × 15 cm^2 heat-sealable pouch and filled with 20 mL of methyl salicylate. Methyl salicylate was chosen as the clearing agent because its refractory index is very close to that of tissue. This resulted in superior photographic resolution with a clean background. The pouch was left overnight; on the next day, it was pressed flat to remove excess methyl salicylate and air.

Digitization of whole mounts and assessment of optical density of mammary epithelium. All whole-mount images were captured by digital photography (Kodak DCS 420, Kodak Digital Science, Rochester, NY) with the light source passing through the sample from underneath the slides. The Kodak DCS 420 is a digital camera that has a spatial resolution of 1.5×10^6 pixels (1012 × 1524) per image. The images were downloaded to Adobe Photoshop (Adobe Systems, San Jose, CA) using a Kodak DCS TWAIN driver. The digitized color images were converted to gray scale (256 shades) images and analyzed by the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). An image-filtering technique was introduced to reduce the intensity variations in the background pixels. Measurements of the mammary fat pad area and the mass of mammary epithelium were performed on the digitized image of the entire abdominal-inguinal mammary gland chain. Mammary fat pad area is defined as the area within the perimeter bounded by circumscribing the outermost terminal end buds of the mammary tree. The mass of mammary epithelium was determined based on the calculation of Σ (a defined area containing mammary epithelial elements × optical density of the assigned area). This integrated value is expressed in arbitrary units of optical density (AUOD). Statistical analysis among groups was done by ANOVA with post-hoc comparisons using Tukey's multiple range test (Zhu et al. 1998).

Quantitation of TEB density. The procedure of preparing mammary whole mounts and staining with alum carmine was similar to that described in the above section. The outer 5-mm margin of the mammary whole mount was examined by light microscopy using the criteria of Russo and Russo (1978). This area represents the location of most of the actively proliferating TEB structures of the mammary gland for a young virgin rat. Images from a minimum of eight fields were transferred from the microscope to a Kodak 8650 PS color printer. Hard-copy pictures were printed out with the use of Adobe Photoshop (Adobe Systems, San Jose, CA). The density of TEB in each field was determined by adjusting the micrometer bar to the power of magnification (Banni et al. 1999). Statistical analysis was done by ANOVA with post-hoc comparisons as described above.

Immunohistochemical staining of PCNA in TEB cells. The proliferating cell nuclear antigen (PCNA) is expressed in early G_1 and S phases of the cell cycle and serves as a good marker for proliferating cells. Methacarn-fixed mammary tissues were processed in a Tissue-Tek Vacuum Infiltration Processor (Miles Scientific, Elkhart, IN) and embedded in paraffin blocks. Ribbons of 5-μm thickness were cut and placed on slides that had been treated with 3-aminopropyl-triethoxysilane. The sections were heat immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol and rinsed in deionized water and then PBS.

Mouse monoclonal PCNA antibody, purchased from Santa Cruz Biotechnology, (Santa Cruz, CA), was used at a dilution of 1:20,000. Tissue sections were exposed to the primary antibody for 1 h at room temperature in a humid chamber. They were then treated with a biotinylated rabbit secondary antibody against mouse immunoglobulin. This was followed by the addition of streptavidin horseradish peroxidase, which binds to biotin. Diaminobenzidine was used as the chromogen to generate a brown precipitate due to its reaction with peroxidase. All slides were counterstained with hematoxylin, rinsed, dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Cells expressing the antigen were identified by a brown stain over the nucleus. Because immunohistochemical staining for a protein is not stoichiometric relative to the amount of protein present, differences in staining were analyzed by using a Kruskall-Wallis rank test as described in detail previously (Zhu et al. 1998).

Mammary carcinogenesis bioassay. Rats in the four different dietary groups were given a single dose (50 mg/kg body weight) of methylnitrosourea (MNU) intraperitoneally at 55 d of age for the induction of mammary tumors. Immediately after carcinogen treatment, all rats were switched to a basal 5% corn oil diet (Ip et al. 1995) without CLA. This diet consisted of 5% corn oil, 20% casein, 65% dextrose, 3.5% AIN-76 mineral mix, 1% AIN-76A vitamin mix, 5% alphacel, 0.3% methionine and 0.2% choline bitartrate. Rats were palpated for mammary gland tumors once a week. The experiment was continued for 24 wk before termination. By this time, the development of palpable tumors had reached a plateau for several weeks across all groups. At necropsy, all tumors were excised and fixed for histological evaluation. Only confirmed adenocarcinomas are reported in the results. Tumor incidences at the final time point were compared by χ-squared analysis, and the total tumor yield was compared by frequency distribution analysis (Horvath and Ip 1983).

Analysis of CLA isomers in tissues. Liver, mammary fat pad, peritoneal fat and plasma were stored at −80°C until they were ready for analysis. Total lipid was extracted by the method of Folch et al. (1957). Free fatty acids were obtained by a mild saponification procedure described by Banni et al. (1994). Methyl esters were prepared by the addition of 14% BF_3/CH_3OH at room temperature and immediately extracted into a solvent consisting of *n*-hexane/water (4:3). After centrifugation at 900 × *g* for 10 min to separate the two phases, the hexane phase was saved and the aqueous phase was further extracted by another round of hexane. The two hexane collections were combined, dried and redissolved in 500 μL *n*-hexane.

Although the BF_3 method causes isomerization of CLA, especially at high temperature (Banni and Martin 1998), we verified the lack of isomerization by BF_3 under the present condition on the basis of the equal analysis of free fatty acid *t,t*-CLA isomers measured in a C-18 column and the methylated *t,t*-CLA isomers measured in a silver-ion column (Banni et al. 1994). Separation of CLA isomers was carried out with a Hewlett-Packard 1050 HPLC system (Hewlett-Packard,

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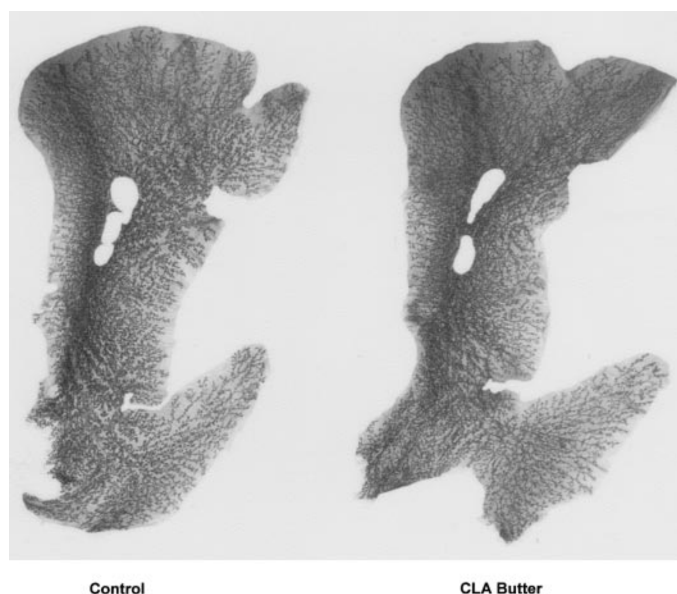

FIGURE 1 Representative mammary gland whole mounts from a control rat or butter conjugated linoleic acid (CLA)-fed rat. The figure shows the entire abdominal-inguinal mammary gland chain from which the lymph nodes had been removed digitally. (Magnification, ×2.3.)

Palo Alto, CA) equipped with a diode array detector 1040M. A silver-ion ChromSpher 5 lipid Chrompack column (Chrompack International BV, Middelburg, The Netherlands), 5-μm particle size, 250 × 4.6 mm, was used with a mobile phase of *n*-hexane with 0.0375% of CH_3CN at a flow rate of 1 mL/min. This technique separates the positional and geometric (*cis* and *trans*) isomers of CLA (Sehat et al. 1998). Conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were stored electronically. Second-derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation (Hewlett-Packard) software. These spectra were taken to confirm the identification of the HPLC peaks. Details of the methodology regarding the characterization of conjugated diene unsaturated fatty acids in both reference and biological samples have been published by Banni and co-workers (1996).

RESULTS

Effect of CLA feeding on mammary epithelial mass and proliferative activity of TEB. CLA feeding had no effect on the growth of the rats. After 1 mo of consuming the different diets starting from weaning, their body weights were: control group, 180 ± 2 g; butter CLA group, 181 ± 2 g; Matreya CLA group, 180 ± 2 g; and Nu-Chek CLA group, 179 ± 2 g. **Figure 1** shows a representative mammary gland whole mount from a rat fed either the control diet or the 0.8% butter CLA diet. Because rats fed a high CLA diet during the 1st mo after weaning developed a less complex network of ductal-alveolar branching regardless of the source of CLA (i.e., butter CLA, Matreya CLA or Nu-Chek CLA), only one example from a high CLA group is presented as an illustration. CLA feeding reduced the development of the mammary tree within the mammary fat pad. The data on epithelial mass of the entire abdominal-inguinal mammary gland chain, as presented in **Table 3**, are expressed in arbitrary unit of optical density (AUOD). There was ~25% lower total mass of mammary epithelium and amount of epithelium per unit area of mammary fat pad in all of the CLA-treated groups compared with the control group ($P < 0.01$). We did not detect any differences in the area of the mammary fat pad among the CLA treatment groups. The results suggest that CLA suppressed mammary branching morphogenesis but did not interfere with the ability of the secondary or subtending ducts to invade up to the boundary of the fat pad.

Our next experiment was designed to determine whether the decrease in mammary epithelial branching was accompanied by a reduction in the density of TEB, which are the primary sites for the chemical induction of mammary adenocarcinomas in the rat model. In control rats, there were an average of 6.3 TEB/mm^2 in the abdominal-inguinal gland at 55 d of age (**Table 4**). CLA feeding resulted in a 30% decrease in TEB density ($P < 0.05$). This magnitude of response was uniform across all of the CLA treatment groups. The proliferative activity of TEB cells was assessed by PCNA immunohistochemistry. Treatment with CLA caused a 25–30% reduction ($P < 0.05$) in the proportion of TEB cells expressing the PCNA antigen.

Modulation of mammary cancer risk by CLA. The mammary carcinogenesis data in rats fed different sources of CLA for 1 mo from weaning and then given a single dose of MNU are summarized in **Table 5**. There was a significant inhibition of both tumor incidence and yield due to pre-MNU feeding of CLA. Overall, CLA treatment decreased mammary cancer risk by ~50%. The different sources of CLA showed similar efficacies as determined by the two variables (tumor incidence

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TABLE 3

Quantitative analysis of mammary epithelium density in whole mounts of rats fed different sources of conjugated linoleic acid (CLA)[1]

Group	CLA in diet	Mammary fat pad area	Mass of mammary epithelium[2]	Epithelial mass per unit area of mammary fat pad[3]
	g/100 g	*cm^2*	*AUOD*	*AUOD/cm^2 × 10^{-3}*
Control	0.1	11.8 ± 0.4	1.34 ± 0.09	113 ± 6
Butter CLA	0.8	11.8 ± 0.4	1.04 ± 0.03*	88 ± 1*
Matreya CLA	0.8	11.5 ± 0.4	0.98 ± 0.08*	85 ± 5*
Nu-Chek CLA	0.8	11.5 ± 0.4	0.98 ± 0.06*	85 ± 4*

1 The digitized image was taken from whole mounts as represented in Figure 1. The data are presented as mean ± SEM, $n = 9$. *$P < 0.01$ compared with the control group.

2 Mammary epithelial mass was measured in arbitrary unit of optical density (AUOD); refer to Materials and Methods section for details of computation.

3 AUOD per unit area of mammary fat pad was computed by dividing mass of mammary epithelium by mammary fat pad area.

TABLE 4

Terminal end bud (TEB) density in mammary gland and proliferating cell nuclear antigen (PCNA) expression in TEB of rats fed different sources of conjugated linoleic acid (CLA)

Group	CLA in diet	TEB density[1]	PCNA positive cells in TEB[2]
	g/100 g	*n/mm²*	*%*
Control	0.1	6.3 ± 0.4	45.2 ± 3.1
Butter CLA	0.8	4.4 ± 0.2*	31.7 ± 2.2*
Matreya CLA	0.8	4.4 ± 0.3*	34.4 ± 2.7*
Nu-Chek CLA	0.8	4.6 ± 0.3*	32.8 ± 2.5*

[1] Results are expressed as means ± SEM, $n = 6$. *$P < 0.05$ compared with control group.
[2] A total of 12–15 TEB structures were counted per rat; each structure consisted of 100–200 cells on a slide.

and total number of tumors) of the bioassay. Thus the data in Tables 3–5 collectively suggest that CLA feeding during pubescent mammary gland development down-regulates -mammary epithelial growth, decreases the population and proliferative activity of the target TEB cells, and therefore reduces mammary cancer risk when the rats are challenged with a carcinogen.

Analysis of CLA isomer incorporation into tissues. The total CLA content in tissues of rats fed different sources of CLA for 1 mo from weaning is reported in **Table 6**. As expected, rats fed a high CLA diet had significantly more total CLA in liver, mammary fat, peritoneal fat and plasma. It is noteworthy to point out that rats given butter CLA generally accumulated more CLA in their tissues compared with those given either Matreya CLA or Nu-Chek CLA. The extra load was particularly marked in mammary and peritoneal fat pads. For example, the increase in total CLA content in the mammary fat pad was fourfold by butter CLA, 2.6-fold by Matreya CLA and 2.9-fold by Nu-Chek CLA. In peritoneal fat pad, it was 6.5-fold by butter CLA, 3.8-fold by Matreya CLA and 2.8-fold by Nu-Chek CLA.

The profiles of individual CLA isomers (μg/mg lipid and % total CLA) in the different tissues are reported in **Tables 7–10**. In all three CLA-treated groups, the major CLA isomer found in the tissues was the *c*9,*t*11-isomer. A significant greater percentage of this isomer was found in tissues from the butter

TABLE 5

Bioassay of mammary cancer prevention in rats fed different sources of conjugated linoleic acid (CLA)[1]

Group	CLA in diet	Tumor incidence	Total tumors
	g/100 g		*n*
Control	0.1	28/30 (93%)	92
Butter CLA	0.8	15/30 (50%)*	43*
Matreya CLA	0.8	16/30 (53%)*	46*
Nu-Chek CLA	0.8	17/30 (57%)*	48*

[1] CLA feeding was started from weaning and continued for 1 mo (i.e. 23–55 d of age). Methylnitrosourea (MNU) was injected into each rat for mammary tumor induction at this point. All animals were switched to a 5% corn oil diet without CLA after MNU administration and were killed 24 wk later.
* $P < 0.05$ compared with control group.

TABLE 6

Total conjugated linoleic acid (CLA) content in tissues of rats fed different sources of CLA

Group	Total CLA content[1]			
	Liver	Mammary fat	Peritoneal fat	Plasma
	μg/mg lipid			
Control butter	2.6 ± 0.3[a]	7.2 ± 0.4[a]	8.8 ± 0.7[a]	5.4 ± 1.4[a]
Butter CLA	15.7 ± 1.1[c]	36.5 ± 2.3[c]	65.9 ± 1.5[d]	23.3 ± 2.8[c]
Matreya CLA	12.5 ± 1.5[bc]	26.2 ± 1.6[b]	42.6 ± 4.0[c]	18.4 ± 3.1[c]
Nu-Chek CLA	10.2 ± 1.2[b]	28.2 ± 1.3[b]	33.4 ± 2.9[b]	12.5 ± 1.0[b]

[1] Results are expressed as means ± SEM, $n = 6$. Values that do not share superscript letters are significantly different ($P < 0.05$).

CLA and the Matreya CLA groups than in tissues from the Nu-Chek CLA group. The pattern essentially reflected the proportion of the *c*9,*t*11-CLA in the various CLA sources, i.e., 92% in butter CLA, 81% in Matreya CLA and 25.3% in Nu-Chek CLA (see Table 2). The *t*,*t*-isomers represented only a small fraction. The *c*,*c*-isomers were also very low (or sometimes undetectable) in the butter CLA and Nu-Chek CLA groups, but were considerably higher in the Matreya CLA group. This was most likely due to the relatively abundant supply of *c*,*c*-isomer in the Matreya CLA (~18%, see Table 2).

In general, the tissue CLA isomer data in the butter CLA and Matreya CLA groups were quite similar, with the exception of the *c*,*c*-isomer results noted above. A few interesting

TABLE 7

Conjugated linoleic acid (CLA) isomer concentration in liver of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	μg/mg lipid			
t,t				
11,13	—	—	—	0.1 (0.6%)
10,12	—	—	—	0.2 (1.3%)
9,11	—	0.3 (1.6%)	0.6 (4.7%)	0.1 (0.9%)
8,10	—	0.1 (0.5%)	0.3 (2.5%)	0.1 (0.7%)
7,9	—	0.1 (0.7%)	—	—
c,t or *t,c*				
11,13	0.1 (2.8%)	0.4 (2.3%)	0.1 (0.8%)	2.1 (20.8%)
10,12	0.2 (8.5%)	—	0.2 (1.5%)	1.6 (16.4%)
9,11	2.0 (76.2%)	13.3 (85.1%)*	8.4 (67.5%)*	4.4 (43.5%)
8,10	0.1 (3.5%)	0.6 (3.8%)	0.4 (3.5%)	1.4 (14.0%)
7,9	0.2 (9.0%)	0.7 (4.2%)	0.2 (1.8%)	—
c,c				
11,13	—	—	—	—
10,12	—	—	0.1 (0.8%)	0.2 (1.8%)
9,11	—	0.3 (1.8%)	2.1 (16.9%)	—
8,10	—	—	—	—
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.
* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

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TABLE 8

Conjugated linoleic acid (CLA) isomer concentration in mammary fat pad of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	µg/mg lipid			
t,t				
11,13	—	—	—	—
10,12	—	—	—	0.3 (1.0%)
9,11	—	0.4 (1.2%)	1.2 (4.6%)	0.4 (1.4%)
8,10	—	—	0.2 (0.7%)	—
7,9	—	—	—	—
c,t or *t,c*				
11,13	—	—	—	5.1 (18.1%)
10,12	—	—	—	3.9 (13.7%)
9,11	6.7 (93.0%)	34.7 (95.2%)*	22.1 (84.4%)*	15.1 (53.6%)
8,10	—	—	0.4 (1.6%)	3.4 (12.2%)
7,9	0.5 (70.0%)	1.3 (3.6%)	—	—
c,c				
11,13	—	—	—	—
10,12	—	—	—	—
9,11	—	—	2.1 (8.2%)	—
8,10	—	—	0.1 (0.5%)	—
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

points with the Nu-Chek CLA results, however, deserve further comment. The bulk of the CLA isomers in tissues from rats fed Nu-Chek CLA was found as the mixed geometric isomers (i.e., *c,t* or *t,c*), and these will be the focus of this discussion. The percentage of the 11,13-isomer in tissues (20.8% in liver, 18.1% in mammary fat, 19.8% in peritoneal fat and 15.2% in plasma) closely resembled the percentage of the 11,13-isomer present in Nu-Chek CLA (17.6%). Similarly, the tissue incorporation of the 8,10-isomer reflected its proportion in the reagent, i.e., 14% in liver, 12.2% in mammary fat, 14.9% in peritoneal fat and 11.5% in plasma, compared with 15.3% in Nu-Chek CLA. In contrast, the percentage of the 10,12-isomer in tissues (16.4% in liver, 13.7% in mammary fat,16.1% in peritoneal fat and 16.0% in plasma) was much lower than the percentage of the 10,12-isomer present in Nu-Chek CLA (36.5%). It appears that the decrease in the 10,12-isomer was compensated for by an increase in the 9,11-isomer. Thus the percentage of the 9,11-isomer in tissues (43.5% in liver; 53.6% in mammary fat; 43.1% in peritoneal fat; and 49.1% in plasma) was generally much higher than the percentage of the 9,11-isomer present in Nu-Chek CLA (25.3%).

Because the mammary gland is the target tissue of interest, the data in Table 8 will be highlighted in an attempt to gain new insight from the analytical information. The emphasis again will be placed on the *c*9,*t*11-CLA because it is the predominant form. The concentrations of this isomer in the mammary tissue were ~34.7 µg/mg lipid in the butter CLA group and 15.1 µg/mg lipid in the Nu-Chek CLA group. However, the cancer protection benefit was similar in the two groups (Table 5). This is an interesting observation and its implication will be discussed below.

DISCUSSION

This study demonstrates convincingly that milk-fat CLA feeding during the time of pubescent mammary gland development down-regulates morphologic maturation of the mammary epithelium and reduces the risk of mammary cancer in this animal model. To our knowledge, this is the first time that naturally occurring CLA in a food form has been shown to have biological activity. A major attribute of using CLA-enriched milk fat is that the biopotency of CLA can be evaluated in the context of a substance present in our everyday diet. Foods are a relatively inexpensive and effective way in which to deliver substances with cancer protective properties. The concept of CLA-enriched foods could be particularly appealing to people who desire a diet-based approach to cancer prevention without making radical changes in their eating habits. As shown in Table 1, the high CLA butter contains more medium-chain fatty acids than the control butter. We do not believe that this is an important contributing factor to the cancer inhibitory effect of CLA-enriched butter because these fatty acids are not known to modulate carcinogenesis in animal models.

Naturally produced CLA consists predominantly of the 9,11-isomer, whereas the synthetic CLA preparation that is most commonly used in laboratory research contains a mixture of positional isomers. On the basis of a number of endpoints, including mammary morphology, proliferative activity and susceptibility to mammary carcinogenesis, we conclude that the 9,11-CLA isomer is at least as potent as the mixture of isomers in modulating these biological responses at the concentration of dietary CLA provided in this study. There is growing information in the literature on the relationship of

TABLE 9

Conjugated linoleic acid (CLA) isomer concentration in peritoneal fat pad of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	µg/mg lipid			
t,t				
11,13	—	0.7 (1.0%)	—	—
10,12	—	—	—	0.4 (1.3%)
9,11	0.1 (1.4%)	0.7 (1.1%)	1.6 (3.8%)	0.4 (1.2%)
8,10	—	—	—	0.2 (0.6%)
7,9	—	—	0.2 (0.5%)	—
c,t or *t,c*				
11,13	—	—	—	6.6 (19.8%)
10,12	0.2 (1.9%)	0.5 (0.7%)	—	5.4 (16.1%)
9,11	7.5 (84.9%)	60.6 (91.9%)*	35.8 (84.0%)*	14.4 (43.1%)
8,10	0.2 (2.0%)	1.3 (2.0%)	0.6 (1.4%)	4.9 (14.9%)
7,9	0.6 (7.2%)	2.2 (3.3%)	—	—
c,c				
11,13	—	—	—	—
10,12	0.1 (1.1%)	—	—	—
9,11	0.1 (1.5%)	—	4.4 (10.3%)	0.4 (1.3%)
8,10	—	—	—	0.2 (0.6%)
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or <0.1%.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

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TABLE 10

Conjugated linoleic acid (CLA) isomer concentration in plasma of rats fed different sources of CLA[1]

CLA isomer	Dietary group			
	Control	Butter CLA	Matreya CLA	Nu-Chek CLA
	μg/mg lipid			
t,t				
11,13	—	—	—	0.1 (1.1%)
10,12	—	—	—	0.1 (0.9%)
9,11	—	0.6 (2.5%)	0.8 (4.5%)	0.1 (1.1%)
8,10	—	0.2 (0.9%)	—	—
7,9	—	—	—	0.1 (0.9%)
c,t or *t,c*				
11,13	—	0.2 (0.7%)	—	1.9 (15.2%)
10,12	—	0.3 (1.3%)	—	2.0 (16.0%)
9,11	4.5 (82.6%)	20.2 (86.7%)*	14.4 (78.3%)*	6.1 (49.1%)
8,10	—	1.0 (4.4%)	—	1.4 (11.6%)
7,9	0.9 (17.4%)	—	—	—
c,c				
11,13	—	—	—	—
10,12	—	—	—	—
9,11	—	0.6 (2.5%)	3.2 (17.2%)	0.4 (2.7%)
8,10	—	0.2 (1.0%)	—	0.2 (1.3%)
7,9	—	—	—	—

[1] The number in parentheses represents the percentage of each CLA isomer of the total CLA. Where no value is presented, the concentration of the CLA isomer was either undetectable or $<0.1\%$.

* Significantly higher than the corresponding value in the Nu-Chek CLA group ($P < 0.05$).

CLA isomer specificity and biological or biochemical effects. At the whole-animal level, the 10,12-CLA isomer has been reported to be responsible for the CLA-induced reduction in body fat accretion in growing mice (Park et al. 1999) and in milk fat synthesis in lactating cows (Baumgard et al. 1999). Park et al. (1999) also showed that in cultured 3T3-L1 adipocytes, *t*10,*c*12-CLA depressed lipoprotein lipase and enhanced glycerol release into the medium. These biochemical responses, however, were not elicited by *c*9,*t*11- or *t*9,*t*11-CLA. On the other hand, recent evidence has indicated that Nu-Chek CLA and Matreya CLA were equally effective in inhibiting growth and inducing apoptosis of rat mammary epithelial cells in primary culture (M. Ip et al. 1999). CLA is a high affinity ligand and activator of peroxisome proliferator–activated receptors (PPAR), a family of transcription factors known to affect gene expression in a tissue-specific manner (Moya-Camarena et al. 1999a). Using a scintillation proximity assay with bacterially expressed human PPARα ligand binding domain, Moya-Camarena and co-workers (1999b) showed a hierarchy of binding affinity for certain CLA isomers in the order of *c*9,*t*11 > *t*10,*c*12 > *t*9,*t*11. In agreement with its high binding affinity, *c*9,*t*11-CLA was also the most efficacious PPARα activator, as determined in a PPARα-reporter gene assay system. This is probably the first study in which the specificity of a CLA isomer is distinguished with the help of molecular tools. Depending on the complexity of the model system, the ability to control for confounding variables and the nature of the assay endpoints, it is not surprising that different conclusions are obtained from the different studies. It does not mean that these conclusions are conflicting. The lesson to be learned is that the data have to be put in the proper perspective so that they can be interpreted appropriately.

On the basis of the analytical data from four different tissues in this study, there appears to be some selectivity in the uptake or incorporation of the *c*9,*t*11-isomer over the *t*10,*c*12-isomer. Studies in lactating dairy cows have also found a similar difference in that the transfer efficiency of a dietary supplement of the 10,12-CLA isomer was only about one half of that observed for the 9,11-CLA isomer (Chouinard et al. 1999). Similarly, in pigs fed a commercial CLA mixture, Kramer et al. (1998) showed that there was a preferential incorporation of the *c*9,*t*11-isomer into liver phospholipids, and of the *c*11,*t*13-isomer into heart lipids. Little information is currently available regarding the biochemical mechanism that regulates the metabolism of the different CLA isomers. Previous research has shown that CLA can be further desaturated and elongated (Banni et al. 1996 and 1999, Sebedio et al. 1997). Some of the ingested CLA is likely oxidized for the production of energy. Future studies should be designed to provide information concerning whether individual CLA isomers are utilized differently via various metabolic pathways.

We have shown that the reduction in mammary cancer risk by CLA under the present experimental conditions is likely due to a decrease in the target cell population, coupled with a lower level of proliferative activity of the target cells. The diminution in TEB density by CLA feeding is consistent with the down-regulation of mammary epithelial branching as determined by digitized image analysis of the whole mount. All of the above variables responded with the same magnitude of change to the different sources of CLA, even though there was more total CLA in the mammary tissue of rats fed butter CLA compared with those fed either Matreya CLA or Nu-Chek CLA. Clearly the 9,11-CLA isomer is biologically active as an anticarcinogen, given the results of the butter CLA. The similarity of the effects of butter CLA and the other two CLA sources suggests that other isomers of CLA may also possess anticarcinogenic activity. However, we cannot rule out the possibility that the 9,11-CLA isomer is already reaching a maximal effect at the tissue level of 9,11-CLA achieved by feeding the Nu-Chek preparation. It would be instructive to carry out dose-response studies with different CLA isomers.

There is one potential advantage to the high CLA milk fat (or butter fat) that merits further attention. Rats consuming butter CLA consistently accumulated more total CLA in their tissues compared with those consuming either Matreya CLA or Nu-Chek CLA. We suspect that this was probably due to the consumption of vaccenic acid (*t*11–18:1) provided by the high CLA butter fat (see Table 1). Vaccenic acid is converted to 9,11-CLA by the Δ9-desaturase enzyme (Griinari and Bauman 1999). The efficiency of this reaction in rodents will be examined in future experiments. Thus, in addition to providing CLA as is, butter fat may also supply the precursor for the endogenous synthesis of CLA. If the rate of endogenous synthesis of CLA is adequate, this pathway may play an important role, which could translate into a potential difference in dose response to CLA-enriched milk (butter) fat vs. synthetically prepared CLA. This represents a new dimension of the project that was not anticipated at its inception. Further studies will be conducted to investigate other attributes of this novel dairy product.

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ACKNOWLEDGMENTS

The authors thank Todd Parsons, Dorothy Donovan, Mary Partridge, Debbie Dwyer, Benjamin Corl, Lance Baumgard, Kim Bukowski, Eric Hallstead and Rose Updike for their technical assistance

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