

cis-9, *trans*-11 CLA Derived Endogenously from *trans*-11 18:1 Reduces Cancer Risk in Rats¹

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ABSTRACT The present study was designed to examine the effects of increasing dietary levels of vaccenic acid (VA) and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) on chemically induced mammary carcinogenesis in rats. Both fatty acids were provided as a natural component in butter fat. The conversion of VA to CLA by Δ 9-desaturase was documented previously in several species, including rats and humans. Specifically, our objective was to determine the relative contribution of dietary VA and CLA to the tissue concentration of CLA and its ability to inhibit the development of mammary carcinomas. A total of 7 diets were formulated with varying levels of CLA and VA. The overall dietary treatment scheme was designed to evaluate the modulation of mammary cancer risk by 1) small increases of CLA in the presence of a low level of VA and 2) more substantial increases of VA against a background of low levels of CLA. As expected, small increases in dietary CLA at the low end of the CLA dose-response range did not reduce tumorigenesis. In contrast, there was a distinct and marked inhibitory response to VA that was dose dependent. The effect of VA was magnified in this experiment because the dose range of VA tested was much broader than that of CLA. Fatty acid analysis showed that the conversion of dietary VA to CLA resulted in a dose-dependent increase in the accumulation of CLA in the mammary fat pad, which was accompanied by a parallel decrease in tumor formation in the mammary gland. The finding confirms that the conversion of VA to CLA is as important for cancer prevention as the dietary supply of CLA. Thus, VA is also anticarcinogenic, and VA and CLA represent functional food components that are present in ruminant fat. J. Nutr. 133: 2893–2900, 2003.

KEY WORDS: • conjugated linoleic acid • vaccenic acid • milk fat • mammary cancer prevention • functional food

Numerous health benefits have been identified with conjugated linoleic acid (CLA)³ isomer mixtures in biomedical studies in animal models (1,2). Beneficial health effects have included reductions in carcinogenesis and atherosclerosis. The major dietary source of CLA is foods derived from ruminants, especially dairy products; in this case, *cis*-9, *trans*-11 CLA is the predominant CLA isomer (3,4). The *cis*-9, *trans*-11 CLA isomer is an intermediate in rumen biohydrogenation of linoleic acid, and it was originally assumed this was its source in ruminants. However, recent studies have demonstrated that the major source of *cis*-9, *trans*-11 CLA in milk fat is endogenous synthesis via Δ 9-desaturase, with *trans*-11 18:1 (vaccenic acid; VA) as the precursor (5–7).

We recently established that *cis*-9, *trans*-11 CLA was anti-carcinogenic in a rat mammary cancer model when it was supplied in a natural form (esterified in butter fat triglyceride) as a food component (8). Interestingly, tissue concentrations

of *cis*-9, *trans*-11 CLA were greater in rats fed a butter that had been naturally enriched with *cis*-9, *trans*-11 CLA compared with rats fed a comparable amount of the same chemically prepared CLA isomer, and we postulated that this difference was related to endogenous synthesis of *cis*-9, *trans*-11 CLA from VA present in the butter. In addition to the importance of endogenous synthesis in dairy cows cited above, the conversion of VA to *cis*-9, *trans*-11 CLA has also been shown in rodents (9,10), pigs (11) and humans (12–14). Banni et al. (10) demonstrated specifically that feeding rats increasing amounts of pure VA resulted in a progressive increase in the tissue concentrations of *cis*-9, *trans*-11 CLA, and this corresponded to reductions in the number of premalignant mammary lesions after exposure to a chemical carcinogen. In the present study, we examined the effects of increasing dietary levels of VA and *cis*-9, *trans*-11 CLA concentrations (present in butter fat) on chemically induced mammary carcinogenesis in rats. Our objective was to determine the relative contributions of VA and *cis*-9, *trans*-11 CLA to the tissue concentration of *cis*-9, *trans*-11 CLA and its ability to inhibit the development of mammary carcinomas.

MATERIALS AND METHODS

Production of experimental butter fats. The dietary treatments in the rodent carcinogenesis experiment were designed to differ in the

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³ Abbreviations used: CLA, conjugated linoleic acid; MNU, methylnitrosourea; TFA, *trans* fatty acid; VA, vaccenic acid.

TABLE 1

Fatty acid composition of control butter and high vaccenic acid/conjugated linoleic acid (VA/CLA) butter

Fatty acid	Control butter	VA/CLA butter
<i>g/100 g fatty acids</i>		
4:0	4.12	3.69
6:0	2.04	1.81
8:0	1.10	0.99
10:0	2.27	2.10
12:0	2.50	2.31
14:0	8.87	8.67
14:1, <i>cis</i> -9	0.74	0.63
15:0	0.75	0.82
16:0	27.62	23.01
16:1, <i>cis</i> -9	1.52	1.33
17:0	0.47	0.45
18:0	11.85	5.09
18:1, <i>trans</i> -6 to 8	0.46	1.21
18:1, <i>trans</i> -9	0.36	0.84
18:1, <i>trans</i> -10	0.67	2.68
18:1, <i>trans</i> -11	1.30	16.28
18:1, <i>trans</i> -12	0.77	2.48
18:1, <i>cis</i> -9	25.35	14.23
18:2, <i>cis</i> -9, <i>cis</i> -12	3.33	2.93
CLA, <i>cis</i> -9, <i>trans</i> -11	0.51	3.76
18:3, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.41	0.46
20:0	0.14	0.13
Other	2.85	4.10

concentration of VA and *cis*-9, *trans*-11 CLA provided as a natural food. To achieve this, we formulated diets for the rats with combinations of butter from two sources. The butter sources were produced by manipulating the diets of dairy cows as previously described (15) under the auspices of the Cornell University Institutional Animal Care and Use Committee. One group of cows was fed a corn-based total mixed ration to produce a control milk fat; a second group of cows was fed the same total mixed ration supplemented with 2 g/100 g sunflower oil and 1 g/100 g fish oil to produce a milk fat that was enriched with VA and *cis*-9, *trans*-11 CLA. Milk was collected and processed from these two groups of cows to manufacture butter as previously described (15). The fatty acid composition of the two sources of butter is presented in Table 1. There were minor differences in concentrations of several fatty acids between the two butter sources, but there were major differences in the concentrations of VA and CLA.

Protocol of animal treatment. Female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC)

at 45 d of age; all subsequent procedures were approved by Roswell Park Cancer Institute Animal Care and Use Committee. Rats were fed the AIN-76 basal diet as described previously (8) for 1 wk to acclimate them to the powdered diet. All rats were injected with a single dose of methylnitrosourea (MNU: 50 mg/kg body) intraperitoneally at 52 d of age for the induction of mammary tumors. Immediately after MNU administration, a total of 210 rats were divided into 7 groups of 30 each and administered the different dietary treatments for the following 24 wk. Throughout this period, rats were palpated weekly to determine the appearance, size and location of mammary tumors. The experiment was terminated at 24 wk after MNU administration. All tumors were excised and fixed for histological examination, and only confirmed carcinomas are reported in data summaries. Randomly selected subsets of rats ($n = 9$) from each treatment were necropsied. The tumor-free inguinal mammary fat pad, a portion of the liver and plasma were retrieved. Tissue and plasma samples were immediately frozen in liquid nitrogen and then stored at -80°C until fatty acid analysis.

Diet formulation and feeding. All seven diets contained a total of 10 g/100 g butter fat, which was derived from the two butter sources either alone or in combination (Table 2). Diet A represented the control group's diet, which contained a low level of both CLA and VA. By using different proportions of the control butter and the VA/CLA-enriched butter, diets E, F and G were formulated with increasing concentrations of VA at 0.73, 1 or 1.6 g/100 g. Because the two sources of butter contained different levels of CLA, diets B, C and D were supplemented with synthetic *cis*-9, *trans*-11 CLA (Natural, Hovdebygd, Norway; 90% purity) so that the total CLA concentration in diet B matched the CLA concentration in diet E, diet C matched diet F, and diet D matched diet G. All diets contained the same amounts of casein, dextrose, mineral mix, vitamin mix, alpha-cel, DL-methionine and choline bitartrate as described previously (16). Food and water were consumed ad libitum. Fresh food was given every 2 d.

Fatty acid analysis. The -80°C samples of liver and mammary fat pad were pulverized at liquid nitrogen temperature. Total lipids were then extracted from pulverized tissues and plasma by the procedure of Hara and Radin (17) using hexane/isopropanol. Fatty acids were methylated according to Christie (18) with modifications. For fat pad lipids, 40 mg was dissolved in 2.0 mL hexane and 40 μL methyl acetate. Methylation reagent (40 μL of 1.0 mol/L sodium methoxide in methanol) was added, the solution was thoroughly mixed and allowed to react at room temperature for 10 min. The reaction was then terminated by the addition of 60 μL of 0.26 mol/L oxalic acid in diethyl ether. Several grains of anhydrous calcium chloride were added, and the mixture was incubated at room temperature for 1 h. An aliquot of the clear hexane supernatant was removed for analysis by GC after centrifugation at $2400 \times g$, 4°C for 5 min. Plasma and liver samples were processed in a manner similar to that for the mammary fat pad. However, because these typically yielded far less lipid, quantities of all reagents for methylation were adjusted to compensate for the reduced yield.

TABLE 2

Design of diets containing control butter and/or high vaccenic acid/conjugated linoleic acid (VA/CLA) butter¹

Diet	Diet proportion			Fatty acid concentration	
	Control butter	VA/CLA butter	Synthetic <i>cis</i> -9, <i>trans</i> -11 CLA	<i>trans</i> -11 18:1	<i>cis</i> -9, <i>trans</i> -11 CLA
	<i>g/100 g</i>			<i>g/100 g diet</i>	
A	10			0.13	0.05
B	10		0.13	0.13	0.18
C	10		0.19	0.13	0.24
D	10		0.32	0.13	0.37
E	6	4		0.73	0.18
F	4	6		1.00	0.24
G		10		1.60	0.37

¹ The basal diet for all treatments consisted of AIN-76 as described in (8).

TABLE 3

Bioassay of mammary cancer prevention in rats fed increasing levels of conjugated linoleic acid (CLA) and/or vaccenic acid (VA)

Treatment ¹	Tumor incidence		Total tumors	
	%	<i>P</i> -value	<i>n</i>	<i>P</i> -value
A	93		91	
B	93		85	
C	83		74	
D	77	0.15 ²	65	<0.05 ²
E	70	<0.05 ³	63	<0.05 ³
F	47	<0.01 ⁴	49	<0.05 ⁴
G	40	<0.01 ⁵	36	<0.05 ⁵

¹ Diets differed in concentration of *cis*-9, *trans*-11 CLA (Treatments A, B, C and D) and VA (*trans*-11 18:1; B vs. E, C vs. F, and D vs. G) as detailed in Table 2. Each treatment group had 30 rats.

² *P*-value for single-factor ANOVA comparing treatments A, B, C and D.

³ *P*-value for linear contrast comparing treatments B and E.

⁴ *P*-value for linear contrast comparing treatments C and F.

⁵ *P*-value for linear contrast comparing treatments D and G.

FAME were analyzed by GC (Hewlett Packard GC system 6890+ with flame ionization detector, Avondale, PA) using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d. with 0.2- μ m film thickness; Varian, Walnut Creek, CA). A programmed temperature run was used to separate FAME. Inlet and detector temperatures were both 250°C. The oven temperature was initially 80°C, then ramped 2°C/min to 190°C and held for 20 min until ramped 5°C/min to 215°C and held for 15 min. The split ratio was 100:1 and hydrogen was used as the carrier gas at 1.0 mL/min. FAME standards were used to identify sample FAME (Nu-Chek-Prep, Elysian, MN).

Statistical analyses. Fatty acid composition data were analyzed statistically by the General Linear Model procedure of SAS (SAS Institute, Cary, NC). For treatments A, B, C and D, a single-factor ANOVA was used to identify the effect of treatment. Differences between treatment means were identified using the PDIF option of the LSMmeans command. Linear contrasts were used to determine differences between treatments B and E, C and F, and D and G. Treatment effects and differences between means were considered significant when *P* < 0.05. Tumor incidence was compared by χ^2 analysis using the Frequency procedure of SAS. Total tumor numbers were compared by frequency distribution analysis (19).

RESULTS

The overall dietary treatment scheme was aimed at evaluating the modulation of mammary cancer risk by 1) small increases of CLA in the presence of a low level of VA and 2) more substantial increases of VA against a background of low

TABLE 4

Composition of fatty acids from liver lipids of rats fed increasing amounts of vaccenic acid (VA) and conjugated linoleic acid (CLA)

Fatty acid	Treatment ¹				<i>P</i> -value ²	Treatment ¹			<i>P</i> -value ³	<i>P</i> -value ⁴	<i>P</i> -value ⁵
	A	B	C	D		E	F	G			
	— g/100 g fatty acids —					— g/100 g fatty acids —					
14:0	0.56	0.54	0.58	0.67	NS	0.52	0.48	0.42	NS	NS	0.02
14:1, <i>cis</i> -9	0.03	0.05	0.05	0.06	NS	0.03	0.02	0.01	NS	0.09	<0.01
15:0	0.14	0.14	0.13	0.15	NS	0.16	0.15	0.14	0.09	NS	NS
16:0	15.92	15.34	15.64	16.64	NS	16.13	14.88	13.91	NS	NS	0.04
16:1, <i>cis</i> -9	2.10	2.08	2.16	2.42	NS	1.88	1.78	1.55	NS	NS	0.02
17:0	0.30	0.31	0.29	0.31	NS	0.33	0.36	0.36	NS	<0.01	<0.01
18:0	24.19	23.88	23.00	21.82	NS	22.61	23.42	23.98	NS	NS	NS
18:1, <i>trans</i> -6 to 8	0.09	0.08	0.08	0.08	NS	0.13	0.15	0.19	<0.01	<0.01	<0.01
18:1, <i>trans</i> -9	0.11	0.12	0.12	0.13	NS	0.17	0.20	0.25	<0.01	<0.01	<0.01
18:1, <i>trans</i> -10	0.05	0.05	0.06	0.06	NS	0.13	0.17	0.25	<0.01	<0.01	<0.01
18:1, <i>trans</i> -11	0.19	0.22	0.23	0.24	NS	1.14	1.66	2.63	<0.01	<0.01	<0.01
18:1, <i>trans</i> -12	0.23	0.28	0.30	0.28	0.10	0.35	0.40	0.41	0.03	<0.01	<0.01
18:1, <i>cis</i> -9	14.46	14.47	14.97	17.20	NS	14.39	12.73	10.73	NS	NS	<0.01
18:2, <i>cis</i> -9, <i>cis</i> -12	3.42	3.84	3.69	3.76	NS	4.51	4.49	4.97	<0.01	<0.01	<0.01
CLA, <i>cis</i> -9, <i>trans</i> -11	0.09 ^b	0.12 ^b	0.13 ^b	0.19 ^a	<0.01	0.61	0.76	1.10	<0.01	<0.01	<0.01
18:3, all <i>cis</i> 6, 9, 12	0.07	0.09	0.07	0.08	NS	0.06	0.09	0.05	NS	NS	NS
18:3, all <i>cis</i> 9, 12, 15	0.05	0.18	0.17	0.11	0.07	0.19	0.09	0.11	NS	0.07	NS
20:0	0.06	0.04	0.03	0.02	NS	0.01	0.02	0.06	NS	NS	0.03
20:1, <i>cis</i> -11	0.09	0.06	0.08	0.08	NS	0.07	0.06	0.08	NS	NS	NS
20:3, all <i>cis</i> 8, 11, 14	1.14	1.16	1.03	1.03	NS	1.21	1.20	1.47	NS	0.06	<0.01
20:4, all <i>cis</i> 5, 8, 11, 14	19.15	19.45	19.58	17.58	NS	16.66	17.12	16.39	0.02	0.04	NS
20:5, all <i>cis</i> 5, 8, 11, 14, 17	0.48	0.52	0.43	0.50	NS	0.71	0.74	1.06	0.03	<0.01	<0.01
22:4, all <i>cis</i> 7, 10, 13, 16	0.21	0.23	0.22	0.23	NS	0.18	0.27	0.17	NS	NS	NS
22:5, all <i>cis</i> 7, 10, 13, 16, 19	0.25	0.29	0.27	0.24	NS	0.30	0.36	0.34	NS	0.01	0.01
22:6, all <i>cis</i> 4, 7, 10, 13, 16, 19	8.12	7.92	7.97	7.16	NS	8.52	9.62	10.83	NS	<0.01	<0.01
Other	8.50	8.53	8.71	8.95	NS	9.00	8.75	8.53	NS	NS	NS

¹ Diets differed in concentration of *cis*-9, *trans*-11 CLA (Treatments A, B, C and D) and VA (*trans*-11 18:1; B vs. E, C vs. F, and D vs. G) as detailed in Table 2. Data represent mean for a subset of 9 rats for each of the seven treatment groups. NS, nonsignificant, *P* > 0.1.

² *P*-value for single-factor ANOVA comparing treatments A, B, C and D. When a treatment effect was significant, treatments were compared by *t*-test and differences indicated by different superscripts (a, b; *P* < 0.05).

³ *P*-value for linear contrast comparing treatments B and E.

⁴ *P*-value for linear contrast comparing treatments C and F.

⁵ *P*-value for linear contrast comparing treatments D and G.

levels of CLA. Therefore, comparisons among treatments A, B, C and D examine the effect of increasing dietary *cis*-9, *trans*-11 CLA concentration while maintaining a constant VA concentration. Comparisons between treatments with identical *cis*-9, *trans*-11 CLA concentrations, but differing VA concentrations involve treatments B vs. E, C vs. F, and D vs. G (Table 2).

Modulation of mammary cancer risk. Examination of the mammary carcinogenesis data indicated that there was a progressive numerical decline ($P = 0.15$) in tumor incidence with increasing dietary intake of *cis*-9, *trans*-11 CLA (Table 3; treatments A, B, C and D). It should be noted that in this design, the dietary CLA level ranged from 0.05 g/100 g in treatment A to 0.37 g/100 g in treatment D. This range is at the low end of the CLA dose-response curve based on our historical data. Thus, the present finding is not unexpected. In contrast, the reduction in tumor incidence was more pronounced with increasing dietary VA (treatments E, F and G). Against this backdrop of the small increases in CLA, there was a distinct inhibitory response to VA that was dose dependent. The effect of VA was magnified in this experiment because the dose range was broadened compared with that of CLA; additionally, the entire dose range of VA was shifted a bit more to the right. The total numbers of tumors for each group were

also examined. It was evident that the tumor yield data closely paralleled the tumor incidence data, thus further confirming the consistency of the results (Table 3).

Fatty acid analysis of tissue and plasma lipids. The fatty acid composition was determined for liver, plasma and mammary fat pad (Tables 4, 5 and 6, respectively). The proportion of most fatty acids was similar for treatments A, B, C and D, whereas tissue and plasma concentrations of *cis*-9, *trans*-11 CLA increased with increasing dietary intake of *cis*-9, *trans*-11 CLA (Fig. 1). The liver concentration of *cis*-9, *trans*-11 CLA was increased >100% across the range of treatments A to D. The fatty acid concentration of *cis*-9, *trans*-11 CLA in both plasma and mammary fat pad was increased by only 38% when treatment A was compared with treatment D. In each tissue, a trend of increasing tissue *cis*-9, *trans*-11 CLA was observed with increasing dietary *cis*-9, *trans*-11 CLA (Fig. 1; bars corresponding to treatments A, B, C and D). The liver lipid concentration of several (n-3) fatty acids was also increased (Table 4; 20:5, 22:5, 22:6), but the basis is unknown.

At the same intake of *cis*-9, *trans*-11 CLA, a progressive increase in the dietary supply of VA (treatments E, F, and G) resulted in even greater *cis*-9, *trans*-11 CLA concentrations (Fig. 1). Increasing VA from 0.13 g/100 g in the diet to 0.73 g/100 g (B vs. E) resulted in three- to fourfold increases in

TABLE 5

Composition of fatty acids from plasma lipids of rats fed increasing amounts of vaccenic acid (VA) and conjugated linoleic acid (CLA)

Fatty acid	Treatment ¹				<i>P</i> ²	Treatment ¹			<i>P</i> -value ³	<i>P</i> -value ⁴	<i>P</i> -value ⁵
	A	B	C	D		E	F	G			
	— g/100 g fatty acids —					— g/100 g fatty acids —					
12:0	0.19 ^a	0.13 ^{ab}	0.12 ^b	0.09 ^b	0.03	0.18	0.14	0.13	NS	NS	NS
14:0	1.59	1.35	1.23	1.11	NS	1.50	1.37	1.36	NS	NS	NS
14:1, <i>cis</i> -9	0.17	0.12	0.12	0.11	0.10	0.12	0.11	0.09	NS	NS	NS
15:0	0.29	0.28	0.26	0.25	NS	0.32	0.30	0.31	0.09	0.04	<0.01
16:0	21.75	21.37	21.49	20.94	NS	22.41	21.06	21.86	NS	NS	NS
16:1, <i>cis</i> -9	3.51	3.66	3.67	3.38	NS	2.90	3.02	3.26	0.08	NS	NS
17:0	0.30	0.29	0.28	0.28	NS	0.35	0.36	0.34	<0.01	<0.01	<0.01
18:0	14.77	14.48	14.18	14.52	NS	14.53	14.67	12.00	NS	NS	0.03
18:1, <i>trans</i> -6 to 8	0.13	0.12	0.12	0.12	NS	0.21	0.23	0.29	<0.01	<0.01	<0.01
18:1, <i>trans</i> -9	0.14	0.15	0.15	0.14	NS	0.24	0.27	0.36	<0.01	<0.01	<0.01
18:1, <i>trans</i> -10	0.15	0.12	0.13	0.12	0.08	0.33	0.38	0.56	<0.01	<0.01	<0.01
18:1, <i>trans</i> -11	0.31	0.28	0.27	0.26	NS	1.82	2.42	3.77	<0.01	<0.01	<0.01
18:1, <i>trans</i> -12	0.32	0.32	0.33	0.31	NS	0.46	0.57	0.79	NS	0.01	<0.01
18:1, <i>cis</i> -9	29.59	30.55	30.97	31.76	NS	27.22	25.85	26.31	0.01	<0.01	<0.01
18:2, <i>cis</i> -9, <i>cis</i> -12	4.36	4.53	4.32	4.36	NS	5.19	4.92	4.82	NS	NS	NS
CLA, <i>cis</i> -9, <i>trans</i> -11	0.24 ^b	0.29 ^a	0.31 ^a	0.33 ^a	<0.01	1.31	1.76	3.39	<0.01	<0.01	<0.01
18:3, all <i>cis</i> 6, 9, 12	0.06	0.08	0.07	0.08	NS	0.05	0.05	0.04	<0.01	0.07	<0.01
18:3, all <i>cis</i> 9, 12, 15	0.11	0.10	0.07	0.08	NS	0.12	0.09	0.11	NS	NS	NS
20:0	0.03	0.03	0.03	0.02	NS	0.02	0.04	0.04	NS	NS	0.10
20:1, <i>cis</i> -11	0.13	0.14	0.16	0.17	NS	0.15	0.13	0.16	NS	NS	NS
20:3, all <i>cis</i> 8, 11, 14	0.62	0.67	0.57	0.62	NS	0.73	0.69	0.71	NS	NS	NS
20:4, all <i>cis</i> 5, 8, 11, 14	9.40	9.54	9.88	9.45	NS	8.36	8.70	6.07	NS	NS	<0.01
20:5, all <i>cis</i> 5, 8, 11, 14, 17	0.27	0.29	0.26	0.29	NS	0.37	0.33	0.40	NS	NS	0.06
22:4, all <i>cis</i> 7, 10, 13, 16	0.09	0.09	0.07	0.04	NS	0.01	0.02	0.01	<0.01	0.06	NS
22:5, all <i>cis</i> 7, 10, 13, 16, 19	0.04	0.08	0.07	0.04	NS	0.10	0.02	0.11	NS	NS	0.07
22:6, all <i>cis</i> 4, 7, 10, 13, 16, 19	2.44	2.46	2.64	2.58	NS	2.92	3.52	3.14	NS	<0.01	0.08
Other	9.01	8.45	8.25	8.57	NS	8.08	8.99	9.56	NS	NS	NS

¹ Diets differed in concentration of *cis*-9, *trans*-11 CLA (Treatments A, B, C and D) and VA (*trans*-11 18:1; B vs. E, C vs. F, and D vs. G) as detailed in Table 2. Data represent mean for a subset of 9 rats for each of the seven treatment groups. NS, nonsignificant, $P > 0.1$.

² *P*-value for single-factor ANOVA comparing treatments A, B, C and D. When a treatment effect was significant, treatments were compared by *t*-test and differences indicated by different superscripts (a, b; $P < 0.05$).

³ *P*-value for linear contrast comparing treatments B and E.

⁴ *P*-value for linear contrast comparing treatments C and F.

⁵ *P*-value for linear contrast comparing treatments D and G.

TABLE 6

Composition of fatty acids from mammary fat pad lipids of rats fed increasing amounts of vaccenic acid (VA) and conjugated linoleic acid (CLA)

Fatty acid	Treatment ¹				P ²	Treatment ¹			P-value ³	P-value ⁴	P-value ⁵
	A	B	C	D		E	F	G			
	— g/100 g fatty acids —					— g/100 g fatty acids —					
12:0	0.75	0.74	0.72	0.77	NS	0.76	0.75	0.69	NS	NS	<0.01
14:0	3.99	4.01	3.90	4.07	NS	4.13	3.97	3.86	NS	NS	0.03
14:1, <i>cis</i> -9	0.48	0.47	0.46	0.46	NS	0.46	0.43	0.41	NS	NS	<0.01
15:0	0.41	0.42	0.42	0.42	NS	0.45	0.44	0.46	NS	NS	<0.01
16:0	27.05	26.68	26.45	26.56	NS	25.53	25.12	24.66	<0.01	<0.01	<0.01
16:1, <i>cis</i> -9	7.12	6.91	7.16	6.68	NS	6.45	6.38	6.36	NS	0.05	NS
17:0	0.28	0.29	0.29	0.29	NS	0.30	0.30	0.31	NS	NS	NS
18:0	4.16	4.19	4.06	4.31	NS	3.94	3.65	3.21	NS	<0.01	<0.01
18:1, <i>trans</i> -6 to 8	0.18	0.18	0.17	0.18	NS	0.28	0.31	0.39	<0.01	<0.01	<0.01
18:1, <i>trans</i> -9	0.22	0.22	0.22	0.23	NS	0.34	0.37	0.44	<0.01	<0.01	<0.01
18:1, <i>trans</i> -10	0.25	0.25	0.26	0.25	NS	0.51	0.62	0.89	<0.01	<0.01	<0.01
18:1, <i>trans</i> -11	0.36	0.37	0.37	0.38	NS	2.15	2.75	4.17	<0.01	<0.01	<0.01
18:1, <i>trans</i> -12	0.40	0.39	0.39	0.38	NS	0.74	0.88	1.10	<0.01	<0.01	<0.01
18:1, <i>cis</i> -9	39.27	39.45	39.64	39.74	NS	35.84	33.59	31.22	<0.01	<0.01	<0.01
18:2, <i>cis</i> -9, <i>cis</i> -12	6.22	5.94	6.22	5.71	NS	5.50	6.29	6.43	NS	NS	NS
CLA, <i>cis</i> -9, <i>trans</i> -11	0.48 ^c	0.57 ^b	0.59 ^b	0.66 ^a	<0.01	2.21	2.85	4.14	<0.01	<0.01	<0.01
18:3, all <i>cis</i> 6, 9, 12	0.04	0.04	0.04	0.04	NS	0.03	0.03	0.04	0.03	0.08	NS
18:3, all <i>cis</i> 9, 12, 15	0.29	0.27	0.29	0.26	NS	0.24	0.27	0.29	NS	NS	NS
20:0	0.06	0.06	0.06	0.07	NS	0.07	0.06	0.06	NS	NS	NS
20:1, <i>cis</i> -11	0.14	0.13	0.14	0.14	NS	0.14	0.15	0.14	NS	NS	NS
20:3, all <i>cis</i> 8, 11, 14	0.03	0.03	0.03	0.03	NS	0.03	0.03	0.04	NS	NS	0.09
20:4, all <i>cis</i> 5, 8, 11, 14	0.26	0.25	0.27	0.25	NS	0.27	0.27	0.25	NS	NS	NS
20:5, all <i>cis</i> 5, 8, 11, 14, 17	ND	ND	ND	ND		ND	ND	ND			
22:4, all <i>cis</i> 7, 10, 13, 16	0.03	0.03	0.04	0.04	NS	0.03	0.02	0.03	NS	<0.01	NS
22:5, all <i>cis</i> 7, 10, 13, 16, 19	0.01 ^b	0.01 ^b	0.03 ^a	0.02 ^b	<0.01	0.02	0.01	0.03	NS	0.02	0.02
22:6, all <i>cis</i> 4, 7, 10, 13, 16, 19	0.05	0.04	0.04	0.04	NS	0.06	0.07	0.07	<0.01	<0.01	<0.01
Other	7.47	8.04	7.75	8.03	0.05	9.51	10.37	10.30	<0.01	<0.01	<0.01

ND, not detected.

¹ Diets differed in concentration of *cis*-9, *trans*-11 CLA (Treatments A, B, C and D) and VA (*trans*-11 18:1; B vs. E, C vs. F, and D vs. G) as detailed in Table 2. Data represent mean for a subset of 9 rats for each of the seven treatment groups. NS, nonsignificant, $P > 0.1$.

² P -value for single-factor ANOVA comparing treatments A, B, C and D. When a treatment effect was significant, treatments were compared by t -test and differences indicated by different superscripts (a, b; $P < 0.05$).

³ P -value for linear contrast comparing treatments B and E.

⁴ P -value for linear contrast comparing treatments C and F.

⁵ P -value for linear contrast comparing treatments D and G.

tissue and plasma *cis*-9, *trans*-11 CLA concentrations ($P < 0.01$). Comparing treatments C and F, increasing dietary VA from 0.13 to 1.0 g/100 g resulted in an approximately fourfold increase in *cis*-9, *trans*-11 CLA concentration in both tissues and plasma ($P < 0.01$). Similarly, large increases in *cis*-9, *trans*-11 CLA were observed when treatments D and G were compared, corresponding to an increase of VA from 0.13 to 1.6 g/100 g ($P < 0.01$). The two butter sources also differed in concentration of other *trans* fatty acids (Table 1), and increasing their dietary supply increased the tissue and plasma concentration of those fatty acids as well (Tables 3, 4 and 5).

The contribution of dietary VA to tissue *cis*-9, *trans*-11 CLA was compared in liver, plasma and mammary fat pad (Fig. 2). To provide perspective, the relationship between VA and *cis*-9, *trans*-11 CLA in the diet was also included (Fig. 2, insert). The individual data points for liver, plasma and mammary fat pad from all treatments are shown with their corresponding regression line. Within each tissue, increasing the supply of VA linearly increased *cis*-9, *trans*-11 CLA concentration. The regression lines show the relative conversion of VA to *cis*-9, *trans*-11 CLA and the increasing concentration of *cis*-9, *trans*-11 CLA when comparing the diet with liver, liver with plasma, and finally the accumulation of *cis*-9, *trans*-11

CLA in the mammary fat pad. There was a relative increase in the proportion of VA converted to *cis*-9, *trans*-11 CLA over the progression from diet to mammary fat pad. The substantial difference in slope between liver and plasma would presumably reflect endogenous synthesis of *cis*-9, *trans*-11 CLA by hepatic $\Delta 9$ -desaturase.

A comparison of the relative transfer of *cis*-9, *trans*-11 CLA from plasma to the mammary fat pad indicated a quadratic relationship between increasing plasma *cis*-9, *trans*-11 CLA and accumulation of *cis*-9, *trans*-11 CLA in the mammary fat pad (Fig. 3). The relationship was linear at lower concentrations and appeared to approach a plateau at 3 to 4 g/100 g *cis*-9, *trans*-11 CLA in plasma.

DISCUSSION

The *cis*-9, *trans*-11 isomer of CLA has been shown to potentially inhibit mammary carcinogenesis in animal models (20), and CLA accumulation in the mammary gland was necessary to observe the anticancer effect (21). In the present study, mammary fat pad concentration of *cis*-9, *trans*-11 CLA increased with increasing dietary concentration of *cis*-9, *trans*-11 CLA. It is clear that dietary VA was very important

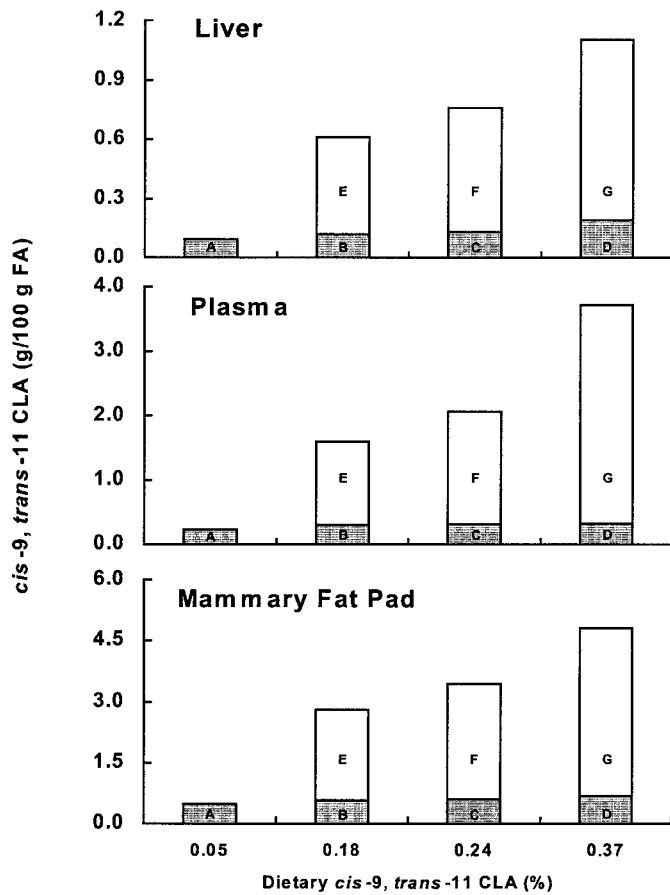


FIGURE 1 Liver (upper panel), plasma (middle panel) and mammary fat pad (lower panel) concentrations of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in rats fed varying concentrations of vaccenic acid (VA; *trans*-11 18:1) and *cis*-9, *trans*-11 CLA. The dietary concentration of *cis*-9, *trans*-11 CLA is shown along the x-axis. Treatments A, B, C, and D contained an identical concentration of VA (0.13 g/100 g) and increasing concentrations of *cis*-9, *trans*-11 CLA (0.05, 0.18, 0.24 and 0.37 g/100 g, respectively). Treatments E, F, and G contained *cis*-9, *trans*-11 CLA that matched diets B, C, and D, respectively and increasing concentrations of vaccenic acid (0.73, 1.00 and 1.60 g/100 g, respectively). Data are means for a subset of 9 rats for each of the seven treatment groups. SEM ranged from 0.015 to 0.084, 0.014 to 0.090, and 0.016 to 0.128 for liver, plasma, and mammary fat pad concentration of *cis*-9, *trans*-11 CLA, respectively.

in determining the CLA concentration of the mammary fat pad. Banni et al. (10) fed VA to rats and showed this increased accumulation of CLA in the mammary gland, resulting in a corresponding reduction of premalignant lesions, an early marker of mammary cancer. The finding from the current study extends these results showing that the additive effect of dietary CLA and conversion of dietary VA to *cis*-9, *trans*-11 CLA caused a dose-dependent increase in the accumulation of CLA in the mammary fat pad and a parallel reduction of total tumor number and tumor incidence. This illustrates the protection afforded by CLA present in the mammary gland and the role of both dietary supply of *cis*-9, *trans*-11 CLA and endogenous synthesis of *cis*-9, *trans*-11 CLA from VA in the diet. The data further reinforce the notion that the conversion of VA to *cis*-9, *trans*-11 CLA is as important for cancer prevention as the dietary concentration of *cis*-9, *trans*-11 CLA, at least in this animal model. We have assumed that the cancer inhibitory effect is wholly attributable to the increased mammary tissue concentration of *cis*-9, *trans*-11 CLA. How-

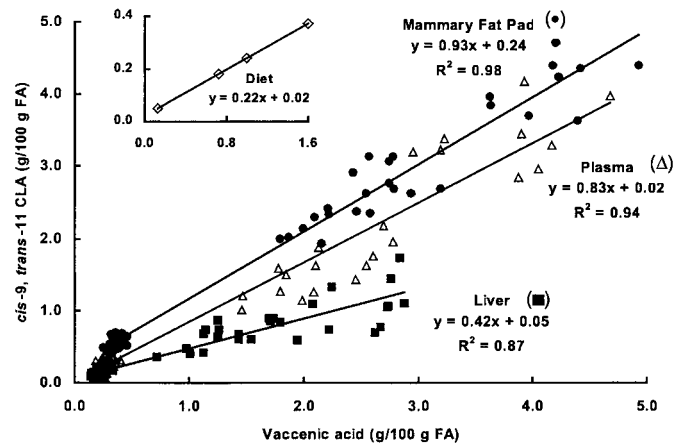


FIGURE 2 The relationship between tissue concentration of vaccenic acid (VA) and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in liver, plasma and mammary fat pad of rats fed varying concentrations of VA (*trans*-11 18:1) and *cis*-9, *trans*-11 CLA. Data points represent individual rats ($n = 9$ for each of the 7 treatment groups) for liver, plasma, and mammary fat pad. For reference, the relationship between VA and CLA in the diet is shown in the inset in which the x-axis and y-axis units are the same as those in the larger graph.

ever, a direct anticarcinogenic effect of *trans*-11 18:1 cannot be completely ruled out because Awad et al. (22) demonstrated that VA was able to modestly inhibit the growth of HT-29 human colon cancer cells compared with stearic acid. Nevertheless, it is clear that VA is anticarcinogenic in this animal model.

The effect of CLA on cancer has been investigated extensively using animal models, and *cis*-9, *trans*-11 CLA has proven particularly effective in preventing mammary cancer [for reviews, see (1,2)]. The mammary gland consists largely of adipocytes and these cells store large amounts of neutral lipids. CLA appears to be preferentially deposited into triglycerides (23), thus explaining its accumulation in the mammary gland. Dietary *cis*-9, *trans*-11 CLA was shown previously to have direct effects on mammary epithelial cells, including decreased proliferation and induction of apoptosis (24–27). Recently,

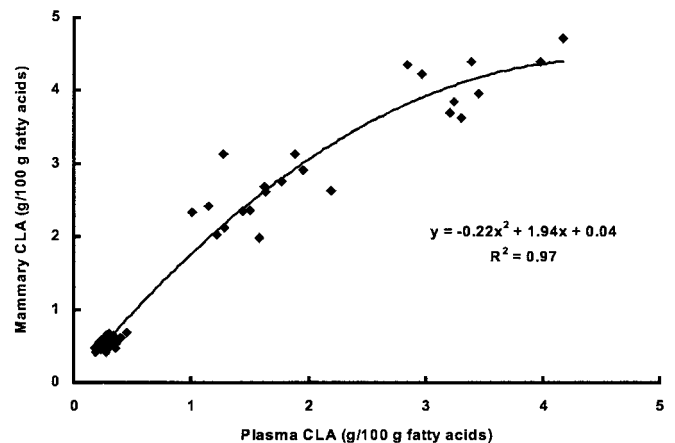


FIGURE 3 The relationship between plasma concentration of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and mammary fat pad concentration of *cis*-9, *trans*-11 CLA from rats fed varying concentrations of vaccenic acid (*trans*-11 18:1) and *cis*-9, *trans*-11 CLA. Each data point represents an individual rat ($n = 9$ for each of the 7 treatment groups).

dietary *cis*-9, *trans*-11 CLA was also shown to prevent the conversion of mammary stromal stem cells to endothelial cells, suggesting a possible role of *cis*-9, *trans*-11 CLA in the inhibition of angiogenesis (28).

The relationship of *cis*-9, *trans*-11 CLA and mammary cancer protection in humans has been examined through epidemiologic studies and results have been inconclusive. Aro et al. (29) found that serum *cis*-9, *trans*-11 CLA was significantly lower in breast cancer cases than controls in postmenopausal women and inferred that the CLA in dairy products could play a protective role against breast cancer. However, Chajés et al. (30) observed no difference in the breast adipose tissue concentration of *cis*-9, *trans*-11 CLA between breast cancer cases and controls. Voorrips et al. (31) used a food-frequency questionnaire to determine intakes of *cis*-9, *trans*-11 CLA and reported a significant relationship between *cis*-9, *trans*-11 CLA and breast cancer incidence (risk ratio of 1.24 after adjusting for known risk factors). However, the fatty acid data used in that study were criticized by others (32). Overall, these limited investigations provide no clear indication of a role for *cis*-9, *trans*-11 CLA in the prevention of breast cancer in humans. In general, differences in the intake of *cis*-9, *trans*-11 CLA between cases and controls, if available, are consistently small and may have been inadequate to observe an effect. The present study (Table 3) and Ip's previous work with the rat model (20,33) demonstrated that there might be a threshold level of CLA for the manifestation of the cancer inhibitory effect. Furthermore, many factors influence breast cancer risk and the ability of *cis*-9, *trans*-11 CLA to alter risk may be dependent on the outcome of genetic and environmental interactions unique to different populations.

The labeling of the *trans* fatty acid (TFA) content of foods has received renewed attention because of the relationship of dietary intake with increases in plasma LDL cholesterol and the risk of coronary heart disease (34). Belury (35) challenged the appropriateness of grouping all TFA into a single entity for food labeling and used CLA isomers as examples of TFA for which beneficial effects have been identified. The present study demonstrates the beneficial effects of *cis*-9, *trans*-11 CLA on tumorigenesis in a rodent model, and further indicates that certain *trans* octadecenoic acid isomers can also have beneficial effects. Food products containing partially hydrogenated vegetable oils are the major dietary source of TFA in the United States (~90% of total). These are predominantly *trans*-18:1 acids, and the partial hydrogenation of vegetable oil produces a Gaussian distribution of *trans* 18:1 isomers that centers on *trans*-9, *trans*-10, and *trans*-11 isomers (36,37). The remainder of dietary TFA comes from food products derived from ruminants, and in this case, the major isomer is VA (36). The present study demonstrates that dietary VA has clear benefits in reducing mammary tumors either through direct action or via its use for endogenous synthesis of CLA. Interestingly, epidemiologic studies have observed a relationship between coronary heart disease risk and dietary intake of TFA from vegetable sources, but no such relationship exists for TFA intake from animal derived foods (38–40).

CLA represents a functional food component because it is present in ruminant fats, and studies with experimental models have identified a number of positive health effects associated with increased CLA intake. Chemically prepared CLA is available as a dietary supplement, and these products contain several CLA isomers. Although some of these formulations are of dubious quality (41), for the most part, they contain an approximately equal proportion of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA as the major ingredients. Recently, there have been two papers demonstrating that *trans*-10, *cis*-12

CLA, but not the *cis*-9, *trans*-11 isomer, induced hyperinsulinemia and insulin resistance in mice (42,43), and that *trans*-10, *cis*-12 CLA, but not a mixed isomer preparation of CLA, induced insulin resistance in obese men (44,45). These very new data confirm the safety of *cis*-9, *trans*-11 CLA, while suggesting that *trans*-10, *cis*-12 CLA should undergo additional study. The production of pure *cis*-9, *trans*-11 CLA pills for the marketplace may be economically unaffordable. On the other hand, VA/CLA-enriched functional food could be a viable way of delivering anticancer agents to the general public. As pointed out earlier, the food form of CLA is predominantly the *cis*-9, *trans*-11 isomer, and VA conversion to *cis*-9, *trans*-11 CLA via Δ^9 -desaturase has been shown to occur in humans (12–14). This is a vanguard preclinical study, which demonstrates the feasibility of this approach.

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