Dietary Conjugated Linoleic Acid Influences the Immune Response of Young and Old C57BL/6NCrlBR Mice.[1,2]

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ABSTRACT Aging is associated with a decline in the immune response in mammals. Conjugated linoleic acid (CLA) has been suggested to have immunoenhancing properties. We examined the influence of dietary CLA on the immune response of young and old mice. Forty young (4 mo) and 40 old (22 mo) mice consumed ad libitum diets containing 0 or 1 g CLA /100 g for 8 wk. Splenocytes from half of the mice were isolated to evaluate proliferation to concanavalin A (Con A) (0.5, 1.5, 5.0 mg/L) and phytohemagglutinin A (PHA) (5, 20, 40 mg/L) and lipopolysaccharide (LPS) (5, 15, 30 mg/L), natural killer cell (NK) activity and prostaglandin (PG)E_2 and interleukin (IL)-2 production. The remaining mice were used to evaluate in vivo delayed-type hypersensitivity (DTH) skin response. There was a significant decline due to age in response to all three mitogens tested ($P < 0.05$). CLA supplementation significantly increased all CLA isomers measured in hepatic neutral lipids and phospholipids ($P < 0.05$). Young mice fed 1% CLA had greater splenocyte proliferation in response to Con A (0.5 and 5.0 mg/L) and PHA (40 mg/L) ($P < 0.05$) than young mice fed control diet. Old mice fed 1 g CLA/100 g had significantly higher proliferative response to optimal concentrations of Con A (1.5 mg/L) ($P < 0.001$) than the mice fed the control diet. Old mice fed the control diet had significantly lower splenocyte IL-2 production than the young mice ($P < 0.005$). CLA-supplemented young mice had significantly higher splenocyte IL-2 production than those fed the control diet ($P < 0.05$). CLA had no effect on NK cell activity, PGE_2 production or DTH in young or old mice. Further studies are needed to determine the mechanism of CLA-induced enhancement of IL-2 production and T cell proliferation. J. Nutr. 129: 32–38, 1999.

KEY WORDS: • *conjugated linoleic acid* • *mice* • *immune response* • *age*

Considerable evidence indicates that aging is associated with altered regulation of the immune system (Miller 1994, Siskind 1980). Age-related functional changes were reported for both humoral and cell-mediated immune responses (Hausman and Weksler 1985, Makinodan 1981, Siskind 1980;). Although all four major cell types of the immune system, i.e. stem cells, macrophages, T cells, and B-cells, show age-related changes, the major alterations were demonstrated in the T cells (Makinodan 1981,Miller and Stutman 1981). In vivo T cell-mediated functions, such as delayed-type hypersensitivity (DTH)[5], resistance to tumors and parasites, and graft vs. host reactions, are depressed with age. Among in vitro indices of T cell-mediated function, the ability of T cells to proliferate in response to antigen or polyclonal T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) and their production of interleukin (IL)-2 were shown to consistently decrease with age (with some exceptions for IL-2).

Intervention with antioxidant nutrients (vitamin E, β-carotene and glutathione) were shown to enhance the immune response in aged rodents and humans (Beharka et al. 1997, Furukawa et al. 1987, Meydani et al. 1986 and 1990, Wu et al. 1994). These nutrients are proposed to exert their immunoenhancing effects through suppression of lipid peroxide and/or prostaglandin (PG) E_2 production (Beharka et al. 1997, Furukawa et al. 1987, Meydani et al. 1986 and 1990).

One potential dietary component that may have an impact on the aging immune response is conjugated linoleic acid

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[5] BHT, butylated hydroxytoluene; *c, cis;* CLA, conjugated linoleic acid; Con A, concanavalin A; cpm, counts per minute; ccpm, corrected counts per minute; DMBA, 2,4-dinitrofluorobenzene; DNFB, 2,4-dinitrofluorobenzene; DTH, delayed type hypersensitivity; FAME, fatty acid methyl esters; [^{125}I] Urd, [^{125}I] iodo-2-deoxyuridine; IL, interleukin; LPS , lipopolysaccharide; NK, natural killer; PG, prostaglandin; PHA , phytohemagglutinin; PKC, protein kinase C; *t, trans.*

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(CLA), a naturally occurring substance originally described as an anti-carcinogenic agent in grilled ground beef. Ha et al. (1987) demonstrated that the application of CLA to mouse skin significantly inhibited the initiation of DMBA [7,12-dimethylbenz(a)anthracene]-induced mouse epidermal tumors. Gavage feeding of CLA also significantly inhibited the initiation of mouse stomach tumorigenesis by benzo(a)pyrene (Ha et al. 1990). Ip et al. (1991) have demonstrated that feeding CLA to mice as 0.5, 1 or 1.5 % of the diet will reduce total tumor incidence and tumor weight of DMBA-induced mammary tumors. In vitro addition of CLA was shown to inhibit cancer cell proliferation (Schultz et al. 1992).

The effect of CLA on the immune system has not been well investigated. When CLA was added to porcine lymphocyte cultures in vitro, it increased mitogen induced lymphocyte blastogenesis, lymphocyte cytotoxic activity and murine macrophage killing ability (Michal et al. 1992). An increase in PHA-stimulated blastogenesis was also noted in CLA fed-chicks challenged with LPS (Cook et al. 1993). In two feeding experiments, CLA added at 0.5% to the diet of chicks or rats decreased the weight loss of these animals in response to *Escherichia coli* LPS challenge (Cook et al. 1993). The authors reported that while CLA had no effect on the animals' response to being injected with the red blood cells of sheep, it did result in an improvement in phytohemagglutin foot pad swelling as well as macrophage phagocytosis in rats.

The mechanism by which CLA exerts its immunological or anticarcinogenic effects has not been well studied. Several investigators have suggested that it may be due to the antioxidant properties of CLA, which were demonstrated in cell free systems (Ha et al. 1990), liver microsomes (Pariza et al. 1991) and in mammary glands (Ip et al. 1991). It was suggested that CLA may be a more potent antioxidant than α-tocopherol and almost as effective as butylated hydroxytoluene (BHT) (Ha et al. 1990). CLA may also exert its effect through alteration of eicosanoid synthesis. PGE_2 was demonstrated to be suppressive to the cellular arm of the immune system (Goldyne and Strobo 1982, Goodwin and Webb 1980, Goodwin et al. 1977). CLA may act in part by competing with linoleic acid in the biosynthesis of arachidonic acid (Ha et al. 1987). In a recent study, Cook et al. (1993) showed that feeding chicks a diet containing 0.5% CLA significantly reduced the level of arachidonic acid in foot pad fatty acids. Also, Belury and Kempa-Steczko (1997) demonstrated that feeding rats 0.5, 1.0 and 1.5% CLA resulted in decreased arachidonic acid levels in hepatic neutral lipids. Arachidonic acid is the precursor for PGE_2; thus, increased CLA intake may decrease PGE_2 production. PGE_2 was shown to have a suppressive effect on IL-2 production and T cell proliferation (Goodwin and Webb 1980, Goodwin et al. 1977). The purpose of the current study was to examine if dietary supplementation with CLA would enhance the immune response of aged mice and to determine if the mechanism of immune enhancement is through alteration of PGE_2 production.

MATERIALS AND METHODS

Animals. Forty specific pathogen-free young (4 mo) and old (22 mo) C57BL/6NCrlBR mice were obtained from NIA colonies at Charles River Laboratories (Kingston, NY). Mice were housed singly in micro-isolator cages at a constant temperature (23°C) with a 12 h light-dark cycle and consumed ad libitum a semi-purified diet supplemented with either 0 or 1 g /100 g CLA (Nu-Check-Prep, Elysian, MN; **Table 1**). The levels of CLA were chosen based on reports in the literature that noted optimal anti-tumor activity at 1 g/100 g CLA of a similar diet as well as on our own preliminary data. The reported isomeric composition of CLA (96% purity) was 43% *cis* (*c*) 9 *trans* (*t*) 11- and *t*9, *c*11 - CLA, 45% *t*10 *c*12-CLA, 6% *c*9 *c*11-*c*10 *c*12, *t*10 *t*12-CLA, 2% linoleate and 4% unidentified compound. CLA was added to the soybean oil prior to its addition to the diet, keeping the total fat at 5 g/100 g. Fat sources were added to the diets on a weekly basis, and diets were stored at 4°C to prevent oxidation. Fresh diets were provided daily, and body weights were monitored weekly. Because previous reports indicated no effect of CLA on the food intake of animals or their weight (Ha et al. 1990, Ip et al. 1991, Zu and Schut 1992), they were not pair-fed. Ten mice from each group were used for in vitro experiments, and 10 mice from each group were used for in vivo DTH experiments. All conditions and handling of animals were approved by the Animal Care and Use Committee at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University and followed NIH Guidelines for the Care and Use of Laboratory animals.

TABLE 1

Composition of the basal diet[1]

Ingredient	*g/100 g*
Vitamin free casein	18.00
Soybean oil	5.00
Corn starch	33.55
Sucrose	33.55
Cellulose	5.00
D-L methionine	0.30
Choline chloride	0.10
Salt mix (AIN-76A)[2]	3.50
Vitamin mix (AIN-76A)[2]	1.00

[1] Ingredients purchased from Harlan Teklad, Madison, WI.
[2] Report of the American Institute of Nutrition (1997).

Tissue collection and cell culture. At the end of the feeding period, mice were killed via CO_2 asphyxiation. Spleens were aseptically removed and placed in 5 mL RPMI 1640 media (Gibco, Grand Island, New York) supplemented with 25 mmol Hepes/L, 2 mmol glutamine/L, 10,0000 U penicillin/L and 100 mg streptomycin/L (Gibco, Grand Island, NY). Livers were then excised, wrapped in aluminum foil, and immediately frozen in liquid nitrogen.

Single cell suspensions were made by teasing spleens between frosted ends of two sterile glass slides. Red blood cells were lysed by Gey's reagent. Cell suspensions were placed in 15 ml sterile conical tubes (Fischer Scientific, Fairlawn, NJ) and were washed two times in 10 ml RPMI at 400 $\times$ *g* for 10 min at 20°C. Cells were counted on a hemocytometer using a light microscope, and viability was determined by trypan blue exclusion method. Cells were adjusted to appropriate concentrations for use in the in vitro assays.

Natural killer cell assay. The natural killer (NK) cell activity against YAC-1 target cells were assessed as previously described (Meydani et al. 1988).

Splenocyte blastogenesis. One hundred μL cell suspension (4 billion cells/L) were added to wells of 96-well plates (Falcon Labware, Lincoln Park, NJ). To these wells 100 μL of media containing either no mitogen, Con A (final concentrations 0.5, 1.5 and 5.0 mg/L), PHA (final concentrations 5, 20 and 40 mg/L) or LPS (final concentrations 5, 15 and 30 mg/L) were added in triplicate. Plates were incubated at 37°C, 5% CO_2 and 100% humidity for 72 h. Cells were then pulsed with 18.5 μBq of [^{3}H]-thymidine for the final 4 h of incubation. The cells were harvested onto glass fiber mats (Wallac, Gaithersburg, MD) by Tomtec harvester (Wallac) and cell proliferation was quantified by the amount of [^{3}H] thymidine incorporation into DNA as determined by liquid scintillation counting in a 1205 Betaplate counter (Wallac). The counter had an efficiency of <50% for ^{3}H. Data are expressed as corrected counts per minute (cpm), which is the cpm of mitogen-stimulated cultures minus the cpm of cultures without mitogen.

Interleukin-1 and interleukin-2 production. For IL-2 production 100 μL of cell suspension at 10 billion cells/L were added to 96-well plates. One hundred μL of media or media containing 20 mg Con A/L or 40 mg PHA/L was added. Cultures were set up in

TABLE 2

Effect of conjugated linoleic acid (CLA) supplementation on body weights of young and old C57BL/6NCrlBR mice fed 0 or 1 g CLA/100 g for 4 wk[1]

Age	CLA	Weight				
		Initial	Week 1	Week 2	Week 3	Week 4
	(g/100 g)	*g*				
Young	0	27.3 ± 0.5[b]	27.8 ± 0.5[b]	28.0 ± 0.6[b]	28.8 ± 0.5[c]	28.9 ± 0.6[b]
	1	26.9 ± 0.5[b]	27.5 ± 0.5[b]	26.8 ± 0.5[b]	26.6 ± 0.5[d]	26.8 ± 0.6[c]
Old	0	38.4 ± 0.9[a]	39.4 ± 0.8[a]	40.2 ± 0.9[a]	41.8 ± 0.9[a]	40.2 ± 1.9[a]
	1	38.9 ± 0.9[a]	40.2 ± 0.9[a]	39.0 ± 0.9[a]	39.1 ± 0.9[b]	39.2 ± 2.0[a]

Data are presented as means ± SEM, $n = 20$. means in a column with different superscripts differ, $P < 0.01$.

quadruplicate. For IL-1 production, 0.5 mL of cell suspension at 10 billion cells/L and 0.5 mL media containing 200 mg LPS/L was added to 24-well plates (Falcon Labware). Plates for both IL-1 and -2 were incubated at 37°C, 5% CO_2 and 100% humidity for 24 h (IL-1, IL-2) at which time they were centrifuged at 400 × *g* at 4°C for 10 min. Supernatants were collected and stored at −70°C until analyzed. IL-1 and −2 concentrations were measured by ELISA kits (Genzyme, Boston, MA) according to the manufacturer's instructions.

Prostaglandin E2 production. Five hundred μL of cell suspension of 10 billion cell/L was added to 24-well plates (Falcon Labware). Five hundred μL of media or media containing 10 mg Con A/L was added to the culture wells. Plates were incubated for 48 h at 37°C, 5% CO_2 and 100% humidity. After the incubation period, plates were centrifuged at 800 × *g* for 20 min at 4°C. Supernatants were collected and immediately frozen at −70°C until analyzed. PGE_2 concentrations were measured by radioimmunoassay as previously described (Hayek et al. 1994).

Delayed-type hypersensitivity. DTH was measured by a previously described radioisotopic method (Vadas et al. 1975). Briefly, mice were sensitized to 2,4-dinitrofluorobenzene (DNFB) by applying 50 μL of a 2% DNFB solution in ethanol on their shaved backs. After 5 d, 10 μL of a 1% DNFB solution in olive oil was painted on both sides of the right ear, and olive oil was applied to the left ear as a control. Ten hours after the second administration of DNFB, mice were injected intraperitoneally with 74 μBq of [^{125}I] iodo-2-deoxyuridine ([^{125}I] Urd) (Amersham, Arlington Heights, IL). Sixteen hours after [^{125}I] Urd injection, the mice were killed and both ears were removed at the hairline and counted in a Cobra II auto-gamma counter (Packard, Meriden, CT). A stimulation index was calculated as:

$$\text{Stimulation Index} = \frac{\text{cpm(right ear)} - \text{cpm(background)}}{\text{cpm(left ear)} - \text{cpm(background)}}$$

Fatty acid analysis. Lipids were extracted with chloroform/methanol (2:1, v/v) and neutral and polar lipids isolated by solid phase extraction (Watkins et al. 1997). Fatty acid methyl esters (FAME) were prepared using 0.5 mol sodium methoxide/ L in anhydrous methanol (Li and Watkins, unpublished results). FAME were analyzed using a gas chromatograph (HP 5890 series II, auto sampler 7673, HP 3365 ChemStation; Hewlett-Packard Co., Avondale, PA) equipped with a DB 225 or DB23 column (30 m, 0.53 mm i.d., 0.5 μm film thickness; J& W Scientific Co., Folsom, CA) and operated at 140°C for 2 min, temperature programmed 1.5°C/min to 198°C and held for 7 min. The injector and flame-ionization detector temperatures were 225 and 250°C, respectively. FAME were identified by comparison of their retention times with authentic standards [GLC-422, CLA (UC-59-A and UC-59-M), Nu-Chek-Prep, Elysian, MN; CLA (Cat. # 1245, *c*9, *t*11 and Cat. # 1181, *t*9, *t*11) Matreya, Inc., Pleasant Gap, PA] and FAME prepared from menhaden oil (Matreya, Inc., Pleasant Gap, PA).

Statistical analysis. Statistical analysis was conducted using the SAS statistical program (Release 6.03, 1988, SAS Institute, Cary, NC). Data were analyzed by a 2 × 2 factorial two-tailed ANOVA with individual differences analyzed by single degree of freedom comparison using Fisher's least significant difference procedure and are reported as means ± SEM. Significance of difference was set at $P < 0.05$.

RESULTS

Old mice had significantly greater body weights than young mice throughout the feeding period (**Table 2**; $P < 0.001$). At Weeks 3 and 4,Young mice fed CLA had lower body weights than those fed the control diet (7% lower, $P < 0.01$). A similar difference was noted in old mice at Week 3 (6% lower; $P < 0.05$). Similar effects of CLA on murine body weights have been reported (Belury and Kempa-Steczko 1997).

CLA-supplemented mice had greater concentrations of CLA 18:2 (9,11) *ct tc*, 18:2 (*t*10,*c*12) 18:2 (*tt*) and total CLA in both neutral lipids and phospholipids (**Table 3;** $P < 0.05$) compared to those fed the control diet. CLA-supplemented mice also had lower total (n-3) fatty acid concentrations and higher (n-6) to (n-3) fatty acid ratios ($P < 0.05$) in neutral lipids compared to those fed the control diet. Incorporation of CLA isomers in the neutral lipids of livers of young mice tended to be greater than in those of old mice ($P = 0.08$). CLA-supplemented young mice also had lower 18:0 concentrations in neutral lipids ($P < 0.05$) compared to those fed the control diet.

Old mice from both diet groups had greater concentrations of 18:1 (n-7) in neutral lipids, lower (n-6) fatty acid concentrations in neutral lipids and lower concentrations of 20:0 in both the neutral lipids and phospholipids (Table 3; $P < 0.05$) compared to young mice.

Splenocyte proliferative responses to all three mitogens were significantly lower in old mice ($P < 0.002$ for Con A and PHA; $P < 0.05$ for LPS; all concentrations) than in young mice. Young mice fed CLA had a greater proliferative response to Con A at 0.5 mg/L ($P < 0.001$) and 5.0 mg/L ($P < 0.05$) (**Fig. 1**) as well as PHA at 40 mg/ L ($P < 0.05$; **Fig. 2**) than those fed the control diet . Old mice fed CLA had significantly higher responses to optimal concentrations of Con A (1.5 mg/L) ($P < 0.001$; Fig. 1) than those fed the control diet. A similar trend ($P < 0.1$) was observed for other concentrations of Con A (0.5 and 5 mg/L) and PHA (5 mg/L). Although CLA had no significant effect on the proliferative response to LPS in young and old mice, there was a significant CLA × age interaction for the LPS 5 mg/L concentration and a trend for the same interaction at the other concentrations (**Fig. 3**; $P < 0.1$).

Interleukin-2 production by splenocytes isolated from old mice was significantly lower than that of young mice

TABLE 3

Effect of conjugated linoleic acid (CLA) supplementation on fatty acid composition of livers of young and old mice.[1,2]

	Neutral Lipids				Phospholipids			
Fatty Acid	Young 0% CLA	Young 1% CLA	Old 0% CLA	Old 1% CLA	Young 0% CLA	Young 1% CLA	Old 0% CLA	Old 1% CLA
				%				
14:0	0.92 ± 0.05^{a}	0.56 ± 0.04^{c}	0.70 ± 0.05^{b}	0.62 ± 0.05^{c}	ND[3]	ND	ND	ND
16:0	24.10 ± 0.72	23.57 ± 0.60	23.04 ± 0.67	24.04 ± 0.67	18.49 ± 0.40	17.98 ± 0.36	17.28 ± 0.40	18.40 ± 0.38
16:1(n-7)	5.61 ± 0.36^{a}	4.12 ± 0.34^{bc}	4.81 ± 0.34^{b}	4.43 ± 0.34^{bc}	1.43 ± 0.14^{a}	0.95 ± 0.12^{b}	1.40 ± 0.14^{a}	1.23 ± 0.13^{ab}
18:0	2.65 ± 0.13^{a}	2.27 ± 0.11^{b}	2.06 ± 0.12^{bc}	1.90 ± 0.12^{c}	15.43 ± 0.52	16.46 ± 0.47	15.92 ± 0.52	16.44 ± 0.49
18:1(n-9)	40.11 ± 1.10^{c}	40.59 ± 0.92^{c}	45.28 ± 1.03^{a}	42.82 ± 1.03^{b}	12.05 ± 0.97^{ab}	10.03 ± 0.87^{b}	13.20 ± 0.97^{a}	12.69 ± 0.92^{a}
18:1(n-7)	4.59 ± 0.28^{b}	4.69 ± 0.23^{b}	5.73 ± 0.26^{a}	5.59 ± 0.26^{a}	2.84 ± 0.19^{bc}	2.48 ± 0.17^{c}	3.89 ± 0.19^{a}	3.09 ± 0.18^{ab}
18:1(total)	44.61 ± 1.32^{c}	45.38 ± 1.10^{bc}	50.64 ± 1.23^{a}	48.41 ± 1.23^{ab}	14.91 ± 1.09^{ab}	12.50 ± 0.98^{b}	16.59 ± 1.09^{a}	15.68 ± 1.03^{a}
18:2								
18:2(9,11)*t,tc*[4]	0.09 ± 0.08^{b}	0.94 ± 0.06^{a}	0.05 ± 0.07^{b}	0.80 ± 0.07^{a}	0.08 ± 0.04^{b}	0.39 ± 0.03^{a}	0.02 ± 0.04^{b}	0.36 ± 0.04^{a}
18:2(t10,c12)	0.01 ± 0.05^{b}	0.53 ± 0.04^{a}	0.00 ± 0.00^{b}	0.42 ± 0.04^{a}	0.01 ± 0.03^{b}	0.30 ± 0.03^{a}	0.00 ± 0.00^{b}	0.23 ± 0.03^{a}
18:2(10,12)*cc*	0.00 ± 0.00^{b}	0.13 ± 0.03^{a}	0.00 ± 0.00^{b}	0.06 ± 0.03^{ab}	0.00 ± 0.00^{b}	0.05 ± 0.01^{a}	0.01 ± 0.01^{b}	0.04 ± 0.01^{a}
18:2(total *tt*)	0.01 ± 0.02^{b}	0.23 ± 0.02^{a}	0.04 ± 0.02^{b}	0.21 ± 0.02^{a}	0.01 ± 0.01^{b}	0.11 ± 0.01^{a}	0.01 ± 0.01^{b}	0.10 ± 0.01^{a}
CLA(total)	0.11 ± 0.15^{b}	1.84 ± 0.13^{a}	0.09 ± 0.14^{b}	1.49 ± 0.14^{a}	0.10 ± 0.08^{b}	0.84 ± 0.07^{a}	0.04 ± 0.08^{b}	0.72 ± 0.07^{a}
18:2(n-6)*c*	12.01 ± 0.84^{a}	11.69 ± 0.70^{a}	8.82 ± 0.78^{b}	10.32 ± 0.78^{ab}	11.96 ± 0.38^{a}	11.01 ± 0.33^{b}	10.24 ± 0.38^{b}	10.36 ± 0.35^{b}
20:0	0.34 ± 0.03^{a}	0.37 ± 0.02^{a}	0.18 ± 0.02^{b}	0.16 ± 0.02^{b}	0.17 ± 0.68^{a}	0.19 ± 0.02^{a}	0.09 ± 0.02^{b}	0.08 ± 0.02^{b}
20:1(n-9)	1.21 ± 0.07^{b}	1.37 ± 0.06^{ab}	1.47 ± 0.07^{a}	1.28 ± 0.07^{b}	ND	ND	ND	ND
20:2(n-6)	0.39 ± 0.04^{b}	0.52 ± 0.04^{a}	0.49 ± 0.04^{ab}	0.44 ± 0.04^{ab}	ND	ND	ND	ND
20:3(n-6)	0.52 ± 0.04^{a}	0.49 ± 0.04^{ab}	0.39 ± 0.04^{ab}	0.37 ± 0.04^{b}	2.54 ± 0.12	2.18 ± 0.11	2.56 ± 0.12	2.37 ± 0.12
20:4(n-6)	1.24 ± 0.15^{a}	1.03 ± 0.13^{ab}	0.77 ± 0.14^{bc}	0.57 ± 0.14^{c}	17.70 ± 0.69	19.37 ± 0.61	18.31 ± 0.68	18.24 ± 0.65
22:5(n-6)	ND[4]	ND	ND	ND	0.54 ± 0.11^{b}	1.00 ± 0.10^{a}	0.54 ± 0.11^{b}	0.76 ± 0.11^{ab}
22:5(n-3)	ND	ND	ND	ND	0.49 ± 0.04^{bc}	0.56 ± 0.03^{ab}	0.46 ± 0.04^{b}	0.63 ± 0.04^{a}
22:6(n-3)	1.35 ± 0.15^{a}	1.07 ± 0.13^{ab}	0.87 ± 0.14^{bc}	0.64 ± 0.14^{c}	12.25 ± 0.60	12.57 ± 0.54	12.19 ± 0.60	11.22 ± 0.57
(n-6)	14.15 ± 0.92^{a}	13.73 ± 0.77^{ab}	10.43 ± 0.86^{c}	11.70 ± 0.86^{bc}	30.54 ± 0.66^{ab}	31.76 ± 0.59^{a}	29.44 ± 0.66^{b}	29.85 ± 0.62^{b}
(n-3)	2.14 ± 0.18^{a}	1.64 ± 0.15^{b}	1.34 ± 0.17^{bc}	1.07 ± 0.17^{c}	12.74 ± 0.61	13.13 ± 0.55	12.65 ± 0.61	11.85 ± 0.58
(n-6)/(n-3), *g/g*	6.82 ± 0.54^{c}	8.77 ± 0.46^{b}	7.98 ± 0.51^{bc}	11.04 ± 0.51^{a}	2.43 ± 0.11	2.42 ± 0.09	2.40 ± 0.11	2.54 ± 0.10

[1] Values are means + SEM, n = 8–10.
[2] Means with no superscripts in common within a row within neutral or phospholipid samples are significantly different, ($P < 0.05$).
[3] ND = Not determined
[4] *c = cis; t = trans*

(**Table 4**; media cultures, $P < 0.002$; Con A-stimulated cultures, $P < 0.0001$). However, IL-2 production in old mice fed 1 g CLA/100 g was not significantly different from that of young mice. Mice fed 1 g CLA/100 g had significantly higher splenocyte IL-2 production (media cultures, $P < 0.01$; Con A-stimulated cultures, $P < 0.01$) than those

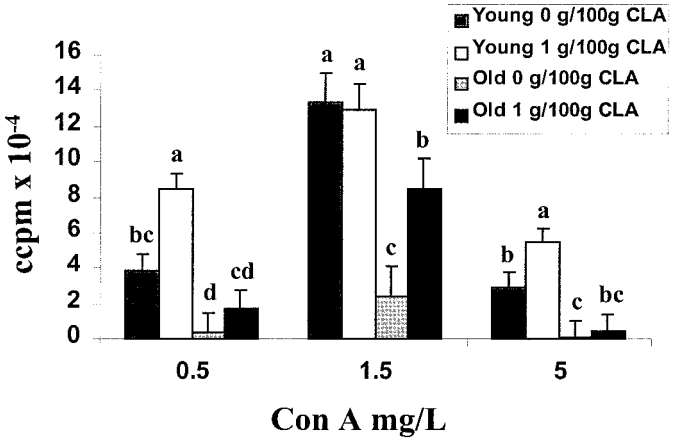

FIGURE 1 Effect of conjugated linoleic acid (CLA) supplemenation on mitogenic response of splenocytes to Concanavalin A (Con A) in young and old C57BL/6NCrlBR mice. One hundred μL of 4 billion splenocytes/L were added to 96-well plates in the presence of 100 μL media or Con A at final concentrations of 0.5, 1.5 or 5.0 mg/L. Plates were incubated at 37°C at 5% CO_2 and 100% humidity for 68 h, and pulsed with 18.5μBq of [^{3}H]-thymidine for an additional 4 h. The cells were harvested on glass fiber strips and [^{3}H]-thymidine incorporation into DNA was determined by liquid scintillation counting. Mitogen-stimulated counts were corrected for background media stimulation (cpm). Data are presented as the means ± SE, n = 10. Bars within a mitogen concentration without common superscripts are significantly different ($P < 0.05$).

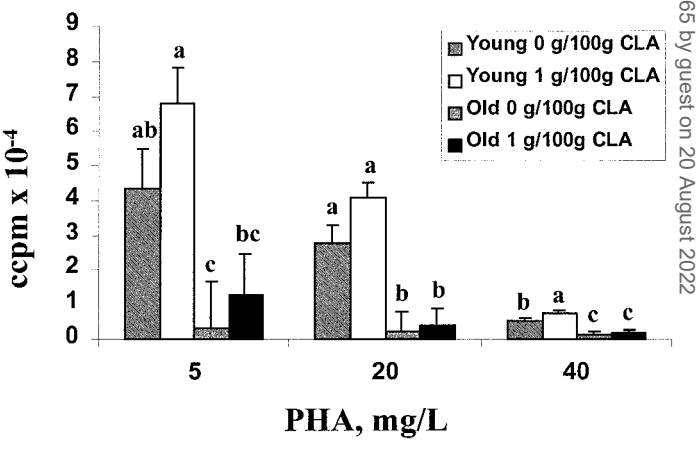

FIGURE 2 Effect of conjugated linoleic acid (CLA) supplemenation on mitogen response of splenocytes to phytohemagglutinin (PHA) in young and old C57BL/6NCrlBR mice. One hundred μL of 4 billion splenocytes/L were added to 96-well plates in the presence of 100 μL media or PHA at final concentrations of 5, 20 or 40 mg/L. Plates were incubated at 37°C at 5% CO_2 and 100 % humidity for 68 h and pulsed with 18.5 μBq of [^{3}H]-thymidine for an additional 4 h. The cells were harvested on glass fiber strips and [^{3}H]-thymidine incorporation into DNA was determined by liquid scintillation counting. Mitogen-stimulated counts were corrected for background media stimulation (cpm). Data are presented as the means ± SE, n = 10. Bars within a mitogen concentration without common superscripts are significantly different ($P < 0.05$).

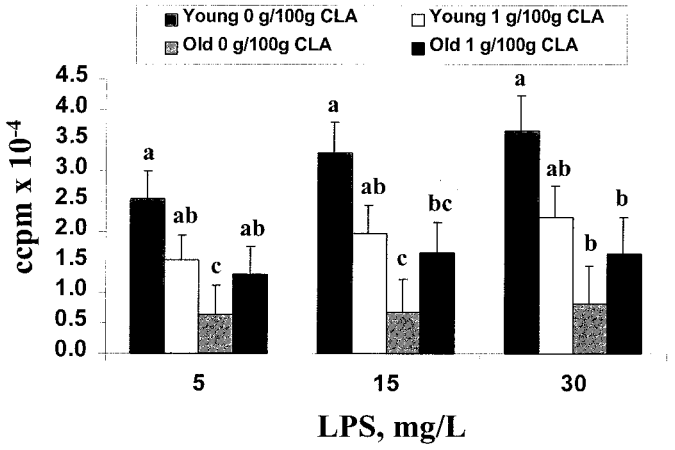

FIGURE 3 Effect of conjugated linoleic acid (CLA) supplementation on mitogen response of splenocytes to lipopolysaccharide (LPS) in young and old C57BL/6NCrlBR mice. One hundred μL of 4 billion splenocytes/L were added to 96-well plates in the presence of 100 μL media or LPS at final concentrations of 5, 15, or 30 mg/L. Plates were incubated at 37°C at 5% CO_2 and 100 % humidity for 68 h and pulsed with 18.5 μBq of [^{3}H]-thymidine for an additional 4 h. The cells were harvested on glass fiber strips and [^{3}H]-thymidine incorporation into DNA was determined by liquid scintillation counting (cpm). Mitogen-stimulated counts were corrected for background media stimulation. Data are presented as the means ± SE, $n = 10$. Bars within a mitogen concentration without common superscripts are significantly different ($P < 0.05$).

fed the control diet. When analyzed as individual treatment means, young mice fed 1 g CLA /100 g diets had significantly higher IL-2 production than the other treatment groups (Table 4). Neither age nor CLA supplementation affected IL-1 production (Table 4).

PGE_2 production from splenocytes was not affected by age or dietary treatment (Table 4). DTH index also did not differ among treatment groups (1.75 ± 0.2, 1.91 ± 0.2, 2.3 ± 0.2, 2.3 ± 02 for Young 0 g CLA/100 g, Young 1 g CLA/100 g, Old 0 g CLA/100 g, Old 1 g CLA/100 g, respectively).

Neither age nor dietary CLA supplementation affected NK activity at any of the effector-to-target ratios tested (data not shown).

DISCUSSION

Conjugated linoleic acid is a mixture of nine isomers with *c*9, *t*11-*t*10, *c*12-, *t*9, *t*11- and *t*10, *t*12-octadecadienoic acids being the four major derivatives that account for more than 90 % of total CLA, whereas the *c*9, *c*11-, *t*9, *c*11-,*c*10, *c*12-, and *c*10, *t*12- octadecadienoic acids represent only minor constituents (Ha et al. 1989). However, only the *c*9,*t*11-CLA isomer was incorporated into the forestomach phospholipid fraction of mice fed CLA (Ha et al. 1990) and the phospholipid fraction of livers and mammary tumors of rats fed CLA (Ip et al. 1991), leading these investigators to conclude that this is the biologically active isomer. In this study, all CLA isomers measured were incorporated into the hepatic neutral lipids and phospholipids of mice fed CLA (Table 3)

Conjugated linoleic acid has been identified in human serum, bile and duodenal juices (Cawood et al. 1983, Fink et al. 1985, Iversen et al. 1985, Szebeni et al. 1986). The potential for increasing CLA concentration in humans was demonstrated in a study that showed that supplementation of cheddar cheese (~112 g/d providing 178.5 mg CLA) increased plasma CLA:LA molar ratio by approximately 130% (Huang et al. 1994). It was also proposed that nonruminants have the ability to produce CLA via the action of intestinal microflora (Chin et al. 1994).

An age-associated decrease in splenocyte blastogenic responsiveness to T cell mitogens Con A and PHA as well as B cell mitogen LPS was noted in this study (Figs. 1–3). Supplemenation of CLA resulted in increased T cell responsiveness to suboptimal and supraoptimal concentrations of Con A and supraoptimal concentration of PHA in young mice, but only optimal concentration of Con A in old mice (Figs. 1 and 2). This is in agreement with Michal et al. (1992) and Chew (1993) who demonstrated that when CLA was added to porcine lymphocyte cultures in vitro, it increased lymphocyte blastogenesis, lymphocyte cytotoxic activity and murine macrophage killing activity. Also, Cook et al. (1993) noted that CLA-supplemented chicks challenged with LPS had higher T cell blastogenic response to PHA. CLA supplementation, however, did not influence in vivo DTH reaction in this study.

An age-associated decline in splenocyte IL-2 production was noted in this study as well. Dietary supplementation with CLA significantly increased splenocyte IL-2 production in young but not old mice. The ability of dietary CLA to increase splenocyte IL-2 production has recently been reported by

TABLE 4

Effect of conjugated linoleic acid (CLA) supplementation on interleukin (IL)-1, prostaglandin (PG) E_2, and IL-2 production from splenocytes of young and old mice[1]

			PGE_2[3]		IL-2[4]	
Age	CLA	IL-1[2]	Media	Con A	Media	Con A
	g/100 g			*μg/L*		
Young	0	60.9 ± 38.2	119.4 ± 15.6	1246.8 ± 293.4	22.2 ± 6.5^b	366.6 ± 69.9^b
	1	68.2 ± 31.9	80.5 ± 13.8	1137.9 ± 245.5	46.2 ± 5.7^a	711.9 ± 58.3^a
Old	0	70.2 ± 38.2	100.5 ± 14.6	840.6 ± 274.5	10.4 ± 5.7^b	182.8 ± 61.4^c
	1	132.3 ± 33.7	153.7 ± 13.8	1648.8 ± 258.8	18.9 ± 5.7^b	242.0 ± 61.4^{bc}

[1] Data are presented as means ± SE, $n = 10$. Means within a column with no superscripts in common are significantly different, $P < 0.01$.
[2] 5 billion splenocytes/L cultured in the presence 100 mg LPS/L. Splenocytes were incubated for 24 h at 37°C at 5% CO_2 and 100% humidity for 24 h.
[3] 5 billion splenocytes/L were cultured in the presence of either media or 10 mg Con A/L for 48 h at 37°C at 5% CO_2 and 100% humidity.
[4] 5 billion splenocytes/L were cultured in the presence of 20 mg Con A/L for 24 h at 37°C at 5% CO_2 and 100% humidity.

others (Wong et al. 1997). In the old mice, however, a numerical increase in IL-2 production was observed but there was no statistically significant difference in IL-2 production by splenocytes of old mice supplemented with CLA and those of young mice fed the control diet. Therefore, supplementation with CLA partially restored decreased IL-2 production in old mice. The reason for the difference in CLA immune enhancement in the different age groups is not clear from this data; however, one potential explanation is the observed trend for lower CLA incorporation into the neutral lipids of old mice in this study.

The effect of CLA on natural killer cell response was examined to determine if the mechanism for its anticarcinogenic properties could be explained through activation of natural killer cell activity. CLA supplementation had no effect on the ability of natural killer cells to kill Yac-1 tumor cells indicating that the anticarcinogenic properties of CLA are not mediated through natural killer cells. Previous studies have suggested that the anticarcinogenic properties of CLA are through inhibition of initiation and promotion stages of carcinogenesis (Belury 1995, Ha et al. 1987, Ip et al. 1997, Liu and Belury 1997) or inhibition of lipoxygenase (Cunningham et al. 1997).

Because CLA can be incorporated into membrane phospholipids and might replace arachidonic acid, it is proposed that CLA can exert its biological effects through the reduction in PGE_2 production (Ha et al. 1990, Ip et al. 1991). In recent studies, Cook et al. (1993) showed that feeding chicks with 0.5 g CLA /100 g significantly reduced the level of arachidonic acid in foot pad fatty acids and that feeding rats with 0.5 g CLA/100 g decreased muscle arachidonic acid levels. Belury and Kempa-Steczko (1997) also demonstrated decreased arachidonic acid levels in hepatic neutral lipids of rats fed 1.0 and 1.5 g CLA/100 g . Because arachidonic acid is a precursor for PGE_2, increased CLA incorporation into cellular membranes may decrease PGE_2 production. We, however, did not observe a significant decrease in arachidonic acid incorporation in hepatic samples or splenocyte production of PGE_2 in young or old mice despite an increase in CLA concentration in both the neutral lipids and phospholipids. This agrees with two recent studies reported that rats fed diets supplemented with 1 g CLA/100 g had increased concentrations of CLA isomers in several tissues and decreased ex vivo PGE_2 production in bone, but not in liver or macrophages (Li et al. 1997, Turek et al. 1997). Also, we did not observe a significant displacement of linoleic acid in the livers of mice fed CLA. This is in agreement with recent data on fatty acid composition of mammary tissue from mice fed CLA (Ip and Schimeca 1997). Thus, CLA replacement of phospholipid arachidonic acid might not be adequate to cause a significant change in PGE_2 production. Further studies are needed to determine whether the effect of CLA on PGE_2 production is species-, organ-, or tissue-specific.

CLA supplementation resulted in lower body weights in both young and old mice. Despite previous reports (Ha et al. 1990, Ip et al. 1991, Zu and Schut 1992) that CLA had no effect on weight, Belury and Kempa-Steczko (Belury 1997) recently reported a weight-reducing effect of CLA. A nutritional intervention that has been demonstrated to enhance immune response is calorie restriction (Umezawa et al. 1990, Weindruch et al. 1982 and 1986). It could be suggested that the immunoenhancing effect of CLA may be through energy restriction. This, however, is not likely as Belury and Kempa-Steczko (1997) reported that CLA resulted in lower body weights in mice without significantly affecting food disappearance, but food wastage was not measured. Also, the beneficial effects of energy restriction on immune response has been observed with an 18% or greater decline in body weight (Umezawa et al. 1990, Weindruch et al. 1986). In our study, only a 7% decline was observed. Lastly, energy restriction needed to be conducted for 6 mo before a beneficial effect on immune response could be observed (Umezawa et al. 1990), whereas the duration of our study was only 1 mo.

Another proposed mechanism for the biological effect of CLA is its potential antioxidant properties. In a cell-free in vitro system CLA was examined for its antioxidant activity by Ha et al. (1990). They observed that under these conditions, CLA was a more potent antioxidant than α-tocopherol and almost as effective as BHT. In another study, Pariza et al. (1991) found that when liver microsomes were subjected to oxidative stress using a non-enzymatic iron-dependent lipid peroxidation system, the microsomes from CLA-treated mice were far more resistant to oxidation than microsomes from control animals. Also, it was reported that feeding CLA to mice from 1 to 6 mo resulted in a decrease in lipid peroxidation in mammary glands but not in the liver, as measured by thiobarbituric acid reactive substances (Ip et al. 1991). However, others have demonstrated that CLA does not act as an antioxidant when tested for its ability to protect membranes composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under conditions of metal ion-dependent or -independent oxidative stress (van den Berg et al. 1995). Our study did not directly examine the antioxidant capability of CLA in these mice. However, in preliminary experiments we noted that CLA had no effect on H_2O_2 production from peritoneal macrophages (data not shown), which suggests a lack of antioxidant function. This is further supported by observations that CLA was more effective in enhancing immune response in young compared to old mice that have been reported to have higher concentrations of free radicals (Sohal and Weindruch 1996).

Other suggested mechanisms for the biological effect of CLA include the inhibition of the induction of ornithine decarboxylase activity by 12-O-tetradecanoylphorbol-13-acetate (Benjamin et al. 1990) as well as potential regulation of protein kinase C (PKC) (Pariza et al. 1991). The former authors suggested that because PKC controls superoxide generation (Merrill 1989), CLA might serve as an antioxidant directly, but indirectly as well (i.e., prevention of superoxide generation via its effect on PKC). Further research is required to determine if these mechanisms can explain CLA's enhancement of T cell proliferation and IL-2 production.

In conclusion, this study further demonstrates that dietary CLA enhances in vitro T cell function but has no effect on in vivo T cell-mediated function as measured by the DTH skin reaction or B cell and NK activity. The immunostimulatory effect was more pronounced in young than in old mice and was not mediated through a change in PGE_2 or IL-1 production. Additional research is required to determine the mechanism of CLA-induced enhancement of IL-2 production and T cell proliferation.

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