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Physiological Difference between Free and Triglyceride-type Conjugated Linoleic Acid on the Immune Function of C57BL/6N Mice

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Previous studies have shown the physiological significance of dietary conjugated linoleic acid (CLA) in various experimental animals and in human beings. One of the important problems to better elucidate is the difference between triglyceride (TG) and free (FFA) dietary CLA. Here, using splenocytes, this study assesses how TG- and FFA-CLA modulate immunoglobulin and various cytokine productions. In this study, C57BL/6N mice were fed an experimental diet containing 0% CLA, 0.1 or 1% FFA-CLA, or 0.1 or 1% TG-CLA for 3 weeks. The production of immunoglobulin tended to be up-regulated by 1% FFA-CLA. As a result of protein array analysis using the supernatant from splenocytes cultured with no CLA, 1% FFA-CLA, and TG-CLA, some cytokine production was shown to be remarkably regulated by dietary FFA- and TG-CLA. A total of 32 cytokines were examined, and 11−14 produced cytokines that were 2-fold up-regulated as compared with control for FFA- or TG-CLA, respectively. Especially, the production of IL-9 and MCP-5 and other cytokines was remarkably up-regulated by both FFA- and TG-CLA. In addition, seven cytokines were 2-fold down-regulated by TG-CLA. These data show that there is a slight but significant difference between the functionalities of FFA- and TG-CLA.

KEYWORDS: Conjugated linoleic acid; triglyceride; free fatty acid; immunoglobulin; cytokine; protein array; mice; splenocytes

INTRODUCTION

Conjugated linoleic acid (CLA) is a generic term for the positional and geometric isomer of octadecadienoic acid. Accumulative data show a diversity of physiological functions such as body fat-reducing (*1*−*3*), antiatherogenic (*4*, *5*), and immunomodulating effects (*6*−*8*). In detail, dietary CLA can up-regulate immunoglobulin (Ig) production from rat and mouse splenocytes and lymphocytes isolated from the mesenteric lymph node (*9*−*11*). Especially in rats, a dose of only 0.05% CLA dramatically enhanced IgA, IgG, and IgM production from rat spleen lymphocytes. In addition, a recent quantum jump in the study of CLA has brought much information concerning the immunomodulatory function (*6*−*11*), and the existence of a more novel effect for CLA on immune function is anticipated.

Commonly, CLA is generated from the isomerization of linoleic acid and supplied as free fatty acid (FFA) (*12*−*14*). Thus, most previous studies on CLA were performed using the FFA type of CLA. On the other hand, most dietary CLA is in the triglyceride (TG) form (*15*, *16*), and, in addition, FFA has a strong astringent taste. Because FFA has an unfavorable taste, application of FFA in various foods is limited. Therefore, examining the physiological functional differences between FFA and TG CLA is an important subject. Here, we examined the levels of immunoglobulin and various cytokines from the cultured supernatant of splenocytes isolated from mice fed FFA- or TG-CLA.

MATERIALS AND METHODS

Experimental Animals and Diets. TG- or FFA-CLA was prepared by Rinoru Oil Mills Co., Ltd. (Nagoya, Japan), as well as high-oleic

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Table 1. Components of the Experimental Diet

	exptl group				
fatty acids (wt %)	control	0.1% FFA	1% FFA	0.1% TG	1% TG
cornstarch	397.48	397.48	397.48	397.48	397.48
casein	200	200	200	200	200
α-cornstarch	132	132	132	132	132
sucrose	100	100	100	100	100
cellulose	50	50	50	50	50
AIN-93G vitamin mix	10	10	10	10	10
AIN-93G mineral mix	35	35	35	35	35
L-cystine	3	3	3	3	3
choline bitartrate	2.5	2.5	2.5	2.5	2.5
TBHQ[a]	0.02	0.02	0.02	0.02	0.02
safflower oil	80	78.7	67.5	78.6	66.0
FFA-CLA	0	1.3	12.5	0	0
TG-CLA	0	0	0	1.4	14.0

[a] *tert*-Butylhydroquinone.

Table 2. Fatty Acid Composition of the Experimental Diet

	exptl group				
fatty acid (wt %)	control	0.1% FFA	1% FFA	0.1% TG	1% TG
16:0	4.5	4.5	4.7	4.5	4.8
18:0	2.0	2.0	2.0	2.0	2.0
18:1	78.0	76.9	67.1	76.8	65.9
18:2 (LA)	13.6	13.4	11.8	13.4	11.6
9*c*,11*t*-CLA	0	0.6	5.7	0.6	6.3
10*t*,12*c*-CLA	0	0.6	5.9	0.7	6.5
other CLAs	0	0.1	0.9	0.1	1.0
18:3	0.3	0.3	0.3	0.3	0.3
20:0	0.4	0.4	0.3	0.4	0.3
20:1	0.3	0.3	0.3	0.3	0.3
others	0.9	0.9	1.1	0.9	1.1

safflower oil. Male, 4-week-old C57BL/6N mice ($n = 40$) (Japan CLEA Inc., Tokyo, Japan) consumed a nonpurified commercial pellet diet and water ad libitum for a week after their arrival. After acclimation, the mice were divided into five groups of eight mice each. They were kept at the Biotron Institute of Kyushu University in a 12-h light/12-h dark cycle (8:00 a.m.−8:00 p.m.) in an air-conditioned room (20 °C and 60% humidity under specific pathogen-free conditions). This experiment was carried out according to the guidelines for animal experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese government. As shown in **Table 1**, experimental diets were manufactured according to the AIN-93G standard containing 0 g/kg CLA (control), 1.25 or 12.5 g/kg FFA-CLA, or 1.40 or 14.0 g/kg TG-CLA. Final CLA percentage was adjusted to 0.1 (0.1% FFA-CLA and 0.1% TG-CLA) or 1% (1% FA-CLA and 1% TG-CLA). Acidic values for TG-CLA and FFA-CLA were 0.80 and 201.3, respectively. For the basic dietary fat source, we used high-oleic-acid safflower oil. The fatty acid composition for these diets is shown in **Table 2**. Blood samples were collected from the tail vein every week, and the serum was prepared and kept at −30 °C before analysis. Body weight was also measured every week. At the end of the feeding period, the liver, spleen, lung, heart, and epididymal and perirenal adipose tissues were excised and weighed. Immediately after the excision, splenocytes were isolated from the spleen.

Isolation of Splenocytes and Culture Condition. Immediately after the excision, splenocytes were isolated from the spleen. Briefly, the spleen was minced and homogenized using a frosted glass, and the remaining scum was removed. Cells were suspended into RPMI1640 medium and washed three times; then red blood cells were lysed using 155 mM NH_4Cl, 10 mM $KHCO_3$, and 10 mM EDTA (pH 7.4). Culture conditions for the splenocytes are appropriately shown for every experimental method.

Measurement of Immunoglobulin Levels by ELISA. To analyze the cytokine and immunoglobulin levels in the culture medium, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed as described in our previous paper (*11*). Splenocytes were cultured in 5% fetal bovine serum containing RPMI1640 medium supplemented with or without 7.8 μg/mL LPS for 24 h, and the culture supernatant was recovered. Rabbit anti-mouse IgA (Zymed, San Francisco, CA), goat anti-mouse IgG (H+L) (Zymed), and rabbit anti-mouse IgM (μ chain specific) (Zymed) were used to fix each Ig. These antibodies were diluted using 10% Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan), added to a 96-well plate, and incubated for 1 h at 37 °C. Three hundred microliters of 25% Block Ace was added and kept at 4 °C overnight, and then samples (50 μL) were added to each well for 1 h at 37 °C. Each well was treated with a solution of either peroxidase (POD)-conjugated goat anti-mouse IgA (Zymed), POD-conjugated goat anti-mouse IgG (H+L) (Zymed), or POD-conjugated rabbit anti-mouse IgM (Zymed) to detect the respective Ig and incubated for 1 h at 37 °C. The plates were rinsed with phosphate-buffered saline (PBS) containing 0.05% polyethylene sorbitan monolaurate (Nacalai Tesque, Kyoto, Japan) between each step. Then, a 10:9:1 mixture of 1.8 mmol/L H_2O_2 in 0.2 mol/L citrate buffer (pH 4.0), H_2O, and 11.7 mmol/L of 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) was added. Finally, absorbance at 415 nm was measured after the addition of 160 mmol/L oxalic acid to stop the coloring reaction.

Protein Array. Cells were cultured in 5% fetal bovine serum containing RPMI1640 medium supplemented with 7.8 μg/mL LPS for 24 h. Thirty-two cytokine proteins were detected using a commercially available mouse cytokine protein array kit (Ray Biotech Inc., Norcross, GA) following the appended protocol. Briefly, mouse cytokine array membranes were incubated with cultured supernatant of splenocytes for 1 h at room temperature, and then the membranes were treated with 250-fold-diluted biotin-conjugated antibodies. Next, membranes were treated with 500-fold-diluted horseradish peroxidase-conjugated streptavidin at root temperature for 1 h. Data were analyzed by using the image analyzer ChemImager 5500 (Alpha Innotech, San Leandro, CA), and the density of each cytokine spot was quantified using a computer program obtained from the U.S. NIH.

Statistical Analysis. Data were analyzed using a commercially available software package according to Fisher's protected least significant difference (PLSD) test method to evaluate the significance of difference. All of the experimental data are shown as means ± SE, and values not sharing any common letter are statistically significant from each other at $p < 0.05$.

RESULTS

Growth Parameters. Because our preliminary study revealed that the food intakes for mice fed the 1% FFA-CLA and 1% TG-CLA diets were lower than control, a pair-feeding study was performed. **Table 3** shows body weight and tissue weight after the experimental period. We set the initial body weight at 16.8−16.9 g for each experimental group, and no significant body weight difference was found for any of the dietary groups. Perirenal and epididymal adipose tissue weights decreased for both the FFA-CLA- and TG-CLA-fed groups in a dose-dependent manner. Potential to reduce adipose tissue weight was comparable for both FFA-CLA and TG-CLA as we could not detect any significant difference between the FFA-CLA and TG-CLA groups at any doses. However, liver weights for the 1% FFA-CLA and 1% TG-CLA groups were significantly higher than the control. In addition, liver enlargement in the 1% TG-CLA group was more remarkable than in the 1% FFA-CLA group. Spleen weight was significantly higher for the 1% FFA-CLA group compared to the control, 0.1% FFA-CLA, and TG-CLA groups. We could not detect any significant difference in the weights for heart, lung, kidney, and spleen among any of the dietary groups.

Immunoglobulin Production from Splenocytes. IgA, IgG, and IgM productivities from splenocytes are shown in **Table 4**. We could detect no significant difference among any of the dietary groups for IgM productivity. On the other hand, 1% FFA-CLA slightly enhanced but 1% TG-CLA slightly inhibited

Table 3. Effect of Conjugated Linoleic Acid on the Growth Parameters of C57BL/6N Mice[a]

	group				
parameter	none	0.1% FFA	1% FFA	0.1% TG	1% TG
body wt (g)					
initial	16.8 ± 1.1	16.8 ± 0.4	16.8 ± 0.7	16.8 ± 0.4	16.9 ± 1.4
final	23.4 ± 1.2	23.1 ± 1.1	22.4 ± 1.1	23.1 ± 0.9	23.6 ± 0.7
tissue wt (g)					
perirenal adipose	0.13 ± 0.01a	0.09 ± 0.01b	0.02 ± 0.00c	0.07 ± 0.01b	0.01 ± 0.00c
epididymal adipose	0.38 ± 0.02a	0.33 ± 0.02b	0.05 ± 0.01c	0.25 ± 0.04b	0.03 ± 0.01c
heart	0.13 ± 0.01a	0.13 ± 0.01a	0.14 ± 0.00ab	0.14 ± 0.01b	0.15 ± 0.00b
kidney	0.37 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.35 ± 0.01	0.37 ± 0.01
liver	1.34 ± 0.05a	1.35 ± 0.06a	1.72 ± 0.07b	1.31 ± 0.03a	2.11 ± 0.09c
lung	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
spleen	0.09 ± 0.01a	0.09 ± 0.00a	0.14 ± 0.02b	0.09 ± 0.01a	0.11 ± 0.01ab

[a] Data are means ± SE for eight mice in each group. Statistical analysis was performed using Fischer's PSLD method; values not sharing a common letter are significantly different from each other at $p < 0.05$.

Table 4. Effect of Conjugated Linoleic Acid on Immunoglobulin Production of Splenocytes Isolated from C57BL/6N Mice[a]

	group				
Ig	control	0.1% FFA	1% FFA	0.1% TG	1% TG
IgA	24.7 ± 3.5ab	22.1 ± 3.9ab	46.2 ± 17.4a	36.8 ± 6.8ab	19.8 ± 2.3b
IgG	28.2 ± 6.5a	27.6 ± 3.8a	83.6 ± 22.8b	74.7 ± 30.3ab	32.7 ± 8.8b
IgM	4.4 ± 0.4	4.0 ± 0.2	5.3 ± 0.8	4.2 ± 0.3	4.5 ± 0.8

[a] Data are means ± SE for three samples in each group. Statistical analysis was performed using Fischer's PLSD test, and no significant difference was detected between all dietary groups at $p < 0.05$. Splenocytes were cultured in 5% FBS–RPMI1640 medium for 24 h. Measurement of the level of immunoglobulin was performed using sandwich ELISA.

IgA productivity, and we could not detect any significant difference between the control and the 0.1% FFA-CLA and 0.1% TG-CLA groups. IgG productivity in the 1% FFA-CLA group was significantly promoted as compared to the control, 0.1% FFA-CLA, and 1% TG-CLA groups; 0.1% TG-CLA was shown to promote IgG productivity, but we could detect no significant difference between the 0.1% TG-CLA and all other remaining dietary groups.

Cytokine Protein Assay. Table 5 shows the results for a cytokine protein array assay on the supernatant taken from cultured splenocytes. The production of some cytokines was shown to be remarkably regulated by dietary FFA- and TG-CLA. Fourteen and seven cytokine productions were up-regulated 2 times compared with control for 1% FFA- and TG-CLA, respectively. Especially, IL-9 and MCP-5 demonstrated significant up-regulation by both FFA- and TG-CLA. In particular, six cytokines, including IL-9 and MCP-5, were shown to be up-regulated over 2 times by both 1% FFA-CLA and 1% TG-CLA. On the other hand, seven cytokines were down-regulated over 2-fold by 1% TG-CLA, and none of the cytokines were down-regulated that much by 1% FFA-CLA as compared to the control group.

DISCUSSION

We previously reported that dietary CLA enhanced IgA, IgG, and IgM production from the rat mesenteric lymph node and spleen lymphocytes (*9*). In rat spleen lymphocytes, only a 0.5 g/kg CLA containing diet could dramatically promot IgA, IgG, and IgM expression (*10*). In addition, the active isomer demonstrated to enhance Ig production was shown to be 10*t*,12*c*-CLA (*11*). These previous studies show that free-type 10*t*,12*c*-CLA is an active isomer to up-regulate immunoglobulin from splenocytes. Generally, it is difficult to apply free fatty acids into foods because of their unfavorable taste. Hence, the comparison of the functionality between FFA- and TG-CLA is an indispensable subject for the general utilization of CLA. As shown in **Table 4**, we affirmed that dietary FFA-CLA at a 1% dietary level promotes Ig productivity of splenocytes. Interestingly, the effect of TG-CLA did not modulate Ig productivity in splenocytes isolated from the 1% diet group, but rather slightly up-regulated IgA and IgG productivities in the 0.1% diet group. From this result, we may anticipate that there is an optimum dose of dietary TG-CLA, which is different from that of FFA-CLA, to enhance Ig production.

How can we eliminate the difference of the functionalities as shown in **Table 4** between FFA- and TG-CLA? It is well-known that dietary TG is destined to be degraded by lipase and decomposed into 2-monoglyceride (MG) and two FFAs. The resulting MG and FFA are efficiently absorbed into the intestinal lumen, and these molecules are resynthesized into TG in epithelial cells. Thus, both dietary TG and FFA are expected to be absorbed into lymphatic vessel in the TG form. In addition, we have already shown that there is no significant difference in the efficiency of lymphatic absorption between FFA-CLA and TG-CLA (unpublished data). Together, these data suggest that there is no significant difference between dietary FFA- and TG-CLA in physiologically. Actually, the body fat reducing potentials of FFA- and TG-CLA were compared, and our results confirm a recent result reported by Terpstra et al. (*17*). In addition, in most studies comparing the effects of FFA- and TG-CLA, no significant difference was found between them (*18*−*20*).

In addition to our data shown in **Table 4**, Rahman et al. reported that dietary FFA could up-regulate the serum glucose level in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, but this effect was not recognized for TG-CLA (*19*). To our knowledge, these are the only reports to show the difference between the two in relation to physiological function. Another putative reason for the difference of functionalities between FFA- and TG-CLA is the structural difference between them

Table 5. Cytokine Protein Array Analysis of the Supernatant Cultured with Splenocytes Isolated from C57BL/6N Mice Fed Free- or Triglyceride-type Conjugated Linoleic Acid

Fold Induction of Cytokines Compared to the Control Group							
1% FFA-CLA				1% TG-CLA			
6Ckine	1.89	IFN-γ	3.49	6Ckine	0.75	IFN-γ	2.61
CTACK	1.74	KC	2.54	CTACK	0.60	KC	1.62
Eotaxin	5.08	Leptin	1.76	Eotaxin	0.83	Leptin	1.43
GCSF	3.42	MCP-1	1.56	GCSF	0.88	MCP-1	0.78
GM-CSF	6.61	MCP-5	13.25	GM-CSF	2.80	MCP-5	15.57
IL-2	3.95	MIP-1a	1.87	IL-2	1.68	MIP-1a	3.02
IL-3	1.61	MIP-2	1.76	IL-3	0.50	MIP-2	1.29
IL-4	1.06	MIP-3β	3.19	IL-4	0.32	MIP-3β	1.80
IL-5	3.67	RANTES	1.12	IL-5	0.39	RANTES	0.37
IL-6	1.65	SCF	3.42	IL-6	0.44	SCF	1.71
IL-9	16.70	sTNFri	4.73	IL-9	6.39	sTNFri	3.32
IL-10	5.57	TARC	1.29	IL-10	2.57	TARC	1.12
IL-12p40	1.28	TIMP-1	1.29	IL-12p40	0.37	TIMP-1	1.03
IL-12p70	1.36	TNF-α	0.98	IL-12p70	0.45	TNF-α	0.88
IL-13	2.09	Tpo	1.35	IL-13	0.89	Tpo	1.09
IL-17	1.90	VEGF	1.58	IL-17	1.23	VEGF	1.62
Up-regulation over 2 Times							
IL-9	**MCP-5**	**GM-CSF**	**IL-10**	**MCP-5**	**IL-9**	**sTNFri**	MIP-1α
Eotaxin	**sTNFri**	IL-2	IL-5	**GM-CSF**	**IFN-γ**	**IL-10**	
IFN-γ	GCSF	SCF	MIP-3β				
KC	IL-13						
Down-regulation over 2 Times							
			IL-3		IL-12p70	IL-6	IL-5
			IL-12p40		RANTES	IL-4	

[a] Data were analyzed by using a commercially available protein array kit. Splenocytes were cultured in 5% FBS–RPMI1640 medium supplemented with 7.8 μg/mL LPS for 24 h. Cytokines presented in bold font with underscore in "Up-regulation over 2 Times" are shown in both FFA- and TG-CLA groups. CTACK, cutaneous T-cell-attracting chemokine; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; KC, CXC ligand 1; MCP. monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; TNF, tumor necrosis factor; TARC, thymus and activation-regulated chemokine; TIMP-1, tissue inhibitor of metalloprotease; Tpo, thrombopoietin; VEGF, vascular endothelial growth factor.

after the intestinal absorption. For example, as a product of lipase-digested TG-CLA, 2-monoglyceride containing CLA at the *sn*-2 position was expected to be the dominant component. To explain the importance of the structure in dietary TG, some researchers pointed out that the structural difference between fish oil and sea lion oil also affected the lipid metabolic function (*21*, *22*). Some structured lipids have been known to modulate various immune functions (*23*−*25*); thus, the structure of dietary TG-CLA deserves consideration. Therefore, even if there is no difference in the lymphatic absorption efficiency between TG- and FFA-CLA, the resulting structure of TG after the resynthesis in intestinal epithelial cells may have important and sequential effects on the immune function.

Finally, results from protein array analysis demonstrate that the effects of dietary FFA- and TG-CLA on various cytokine productions are not exactly the same. As a typical example, dietary TG-CLA strongly down-regulated several LPS-induced cytokine productions, and some cytokines were strongly up-regulated only by FFA-CLA. These data also show the difference in the immunological functions between FFA- and TG-CLA. Modulation of cytokine production, especially IL-2 and interferon-γ, from various kinds of cell types by CLA was shown in some papers (*7*, *11*, *26*, *27*), but to the best of our knowledge, this is the first finding demonstrating that dietary CLA modulates IL-9 and MCP-5 production. IL-9 is produced from CD4 positive T cells and promotes differentiation of erythroblast and proliferation of activated T cell, mast cell, and fetal thymic cells. MCP-5, one of the chemokines, is related to many pathophysiologies (*28*), and the physiological role for this chemokine is now becoming clear. Not only IL-9 and MCP-5 but also some other cytokines, which have never been associated with CLA before, have been shown here to be regulated by CLA. However, currently the physiological significance of these newly found cytokines remains obscure. Therefore, additional study is needed to elucidate this subject, and the results may yield important clues to a fuller understanding of the function of CLA.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; LA, linoleic acid; CTACK, cutaneous T-cell-attracting chemokine; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; KC, CXC ligand 1; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T cell expressed and secreted; SCF, stem cell factor; TNF, tumor necrosis factor; TARC, thymus and activation-regulated chemokine; TIMP-1, tissue inhibitor of metalloprotease; Tpo, thrombopoietin; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TG, triglyceride; FFA, free fatty acid; MG, monoglyceride; ELISA, enzyme-linked immunosorbent assay.

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