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## A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis

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**Butz DE, Li G, Huebner SM, Cook ME.** A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. *Am J Physiol Regul Integr Comp Physiol* 293: R669–R676, 2007. First published June 6, 2007; doi:10.1152/ajpregu.00005.2007.—A naturally occurring fatty acid, conjugated linoleic acid (CLA), reduces immune-induced TNF and inducible cyclooxygenase (COX-2) expression; key mediators of inflammation in rheumatoid arthritis (RA). On the basis of previous work, it was hypothesized that dietary CLA would act as an anti-inflammatory agent in select animal models of RA. In the collagen antibody-induced arthritis (CAIA) model, mice fed CLA (mixed isomers of c9, t11, and t10, c12-CLA) for 3 wk before anticollagen antibody injection had reduced lipopolysaccharide-induced plasma TNF levels and had arthritic scores that were 60% of mice fed corn oil (CO). In the collagen-induced arthritis (CIA) model, mice fed mixed isomers of CLA for 21 days before immunization had lower IgG<sub>1</sub> titers, earlier signs of joint inflammation, but similar arthritis scores compared with CO fed mice during the remaining 70-day post-injection period. Beginning on day 80 to 133, CLA-fed mice had arthritic scores 70% that of the CO-fed mice. In a second CIA experiment, CLA was fed only after the booster injection. Plasma IgG<sub>1</sub> levels were not reduced and arthritis onset was delayed 4 days in CLA-fed mice compared with the CO-fed mice. Peak arthritis score was similar between CLA and CO-fed mice from day 35 to 56. Because CLA reduced inflammation in the CAIA model, delayed onset of arthritis in the CIA model (CIA *experiment 2*) and reduced arthritis score after day 80 in the CIA model (CIA *experiment 1*), we concluded that dietary CLA exhibited anti-inflammatory activity that was dependent on antibody.

collagen-induced arthritis; autoantibody; tumor necrosis factor

CONJUGATED LINOLEIC ACID (CLA) isomers are naturally occurring fatty acids (C18:2) derived primarily from microbial biohydrogenation of linoleic (C18:2) and linoleic acid (C18:3) (6, 26). However, CLA synthesized by alkali isomerization has been the primary source for assessment of its immune modifying properties. Dietary CLA (predominantly equal parts c9, t11 and t10, c12 CLA) was shown to alleviate clinical signs of inflammatory diseases such as atherosclerosis (23, 38), lupus (55, 57), type I airway hypersensitivity (51, 52), endotoxin-induced cachexia (8, 31), and models of inflammatory bowel disease (2, 18). Potential anti-inflammatory properties of CLA may be attributable to its regulation of TNF and inducible cyclooxygenase (COX-2). Dietary CLA reduced LPS or dextran sulfate-induced release of TNF (2, 56) and inducible nitric oxide synthase (56), and antigen-induced release of eicosanoid products of COX-2 (52). Signaling pathways for TNF and

COX-2 such as peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (58), NF- $\kappa$ B (2, 24), and MAPK (25, 41) have been implicated to be affected by CLA.

Rheumatoid arthritis (RA) is a type III hypersensitivity immune disorder (like lupus) characterized by articular joint inflammation and remodeling leading to severe pain and disability (42). Collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis (CIA) are animal models of RA that have been extensively studied (34, 48–50) and have been shown to be suitable for providing insights into the basic pathological mechanisms of chronic inflammation (27). CAIA is the simplest model of RA and involves passive transfer of monoclonal antibodies directed at arthritogenic epitopes of the type II collagen molecule (CII). When these anti-CII antibodies are injected into mice, a severe erosive arthritis similar to RA occurs (49). Unlike the CIA model, the CAIA model eliminates the need for acquired immunity, and results in joint inflammation within 24 h after initiation (47). In the CIA model of RA, articular joint inflammation appears after repeated immunization with CII (12, 50), a putative autoantigen in RA (21, 43), and through the priming of the adaptive immune response (12, 50). Following immunization against CII, susceptible mice develop a severe erosive articular joint destruction similar in pathology to RA (34). Autoantibodies against CII play a key role in joint destruction in both the CAIA and CIA models of RA (44, 49).

Inflammatory mediators such as COX-2 and TNF play important roles in the pathology of RA, CAIA, and CIA. Myers et al. (33) reported that DBA/1 COX-2 knockout mice are resistant to CAIA and CIA, whereas COX-1 (associated with housekeeping functions) knockout, and wild-type mice were shown to develop clinical signs of arthritis as expected. Blockade of the COX pathway using NSAIDs was an effective treatment of acute and chronic inflammation such as in RA (42). The overexpression of the human TNF transgene in arthritis-susceptible mice led to the spontaneous development of severe erosive arthritis similar to CIA and RA (3). Inflammation in both RA and its animal models can be attenuated by COX inhibitors (30) or by anti-TNF therapies (28, 53). Anti-TNF antibody administration in CIA model ameliorated disease (53), and several humanized anti-TNF therapeutics are now approved for RA therapy (14, 15).

On the basis of evidence that dietary CLA reduces inflammatory mediators, such as TNF and COX-2 and inhibiting these mediators decreases autoimmune-induced inflammation of articular joints in models of RA, we hypothesized that

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dietary CLA would reduce inflammation induced by anti-CII antibodies in articular joints. Previous experiments involving the autoimmune disease systemic lupus erythematosus (55, 57) suggested that dietary CLA's effects on adaptive immune responses may confound an interpretation of CLA's role in inflammation. Hence, murine models of inflammation that were dependent or independent of an acquired immune response to CII were used.

## MATERIALS AND METHODS

All studies were conducted in accordance with protocols approved by the College of Agricultural and Life Sciences Animal Care and Use Committee. Figure 1 illustrates the timeline for each of the three experiments performed in the current work.

**Anticollagen antibody-induced arthritis (CAIA) model.** Four-week-old male BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a small laminar flow animal isolator in littered shoebox units (3 mice per unit) with a 12:12-h light-dark cycle. After 3 days of acclimation to the environment, mice were

switched to a semipurified 99% complete diet (TD94060; Harlan-Teklad, Madison, WI) supplemented with 0.5% CLA (mixed isomers) and 0.5% corn oil (CO) or 1% CO (Table 1;  $n = 12$  per diet). CLA and CO fatty acid content (Table 2) was assessed by gas chromatography using an HP-88 100-m fused silica capillary column (0.25 mm inside diameter, 0.2- $\mu$ m film thickness) as previously described (22).

After 3 wk of dietary supplementation, dietary groups were further divided into monoclonal antibody and sham-injected groups to complete a two-by-two factorial design ( $n = 6$  per group, 24 total). On day designated zero (Fig. 1A), mice were injected intravenously (via the tail vein) with 100  $\mu$ l of either 10 mg/ml antibody cocktail (4 monoclonal antibody clones against distinct CII epitopes (Chemicon International, Temecula, CA) or vehicle (sterile PBS). Twenty-four hours later, all mice were injected intraperitoneally with 50  $\mu$ g LPS in PBS (1 mg/ml). Blood was collected from the retroorbital venous plexus, while animals were under light isoflurane anesthesia 1 h after LPS injection. Plasma was separated and samples were stored at  $-80^{\circ}\text{C}$ . Mice were examined daily by a trained observer (unaware of treatment) for evidence of inflammation and joint swelling. The inflammation (arthritis) score was based on a scale of 0 to 4 using the

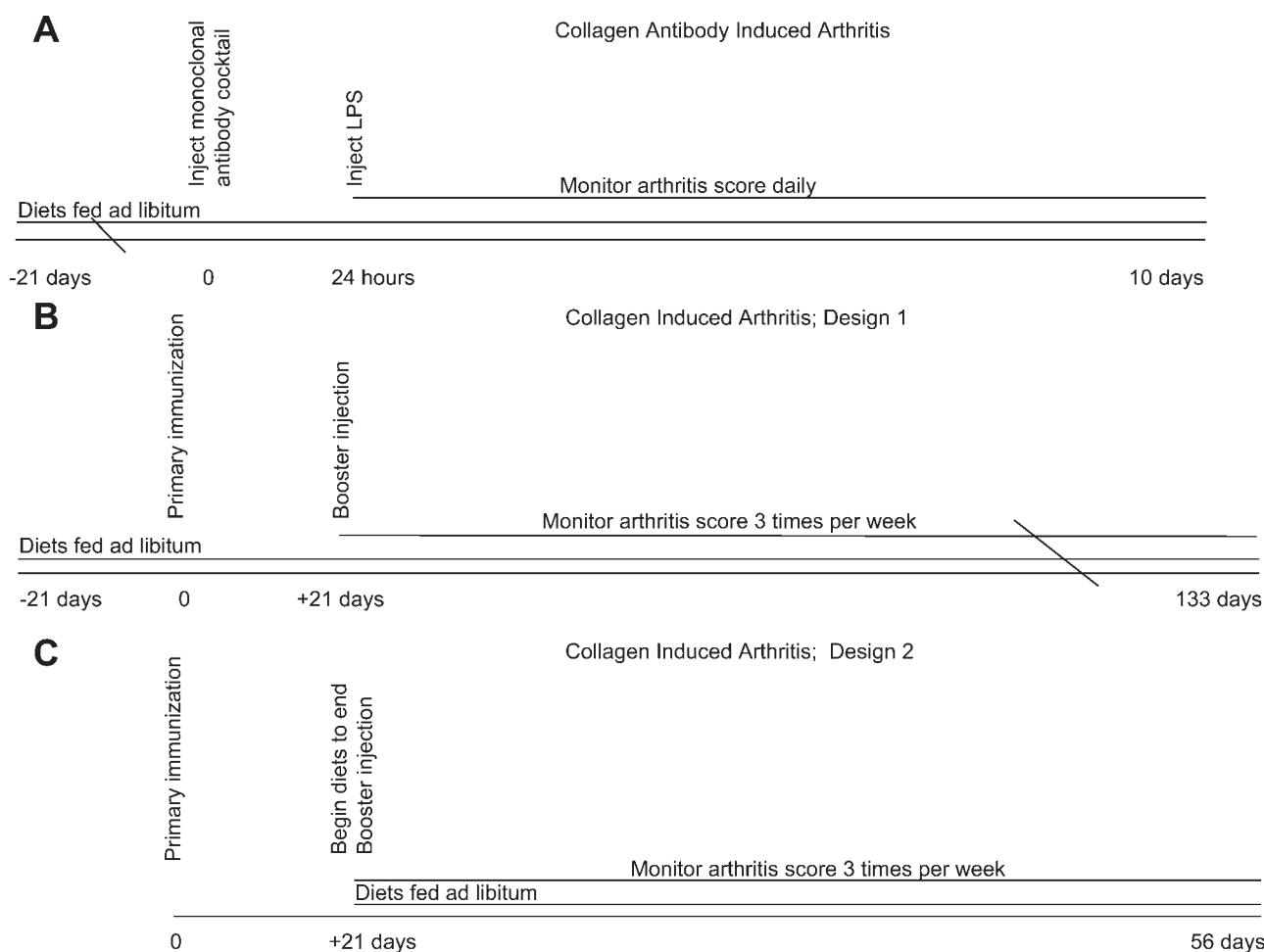


Fig. 1. Illustration of experimental designs and timeline used in each experiment. *A*: collagen antibody-induced arthritis (CAIA) study was conducted as a two-by-two factorial arrangement of treatments with diet (conjugated linoleic acid, CLA or corn oil, CO) and injection (antibody cocktail, or vehicle) as main effects. Diet feeding began 21 days before the injection protocol, and arthritis was scored daily following the completed injection protocol. *B*: first collagen-induced arthritis (CIA) experiment (*design 1*) was conducted as a two-by-two factorial arrangement of treatments with diet (CLA or CO) and immunization type II collagen molecule [(CII)-immunized or sham-immunized] as the main effects. Diet feeding began 21 days before the immunization protocol, and arthritis was scored every 2 or 3 days after the initial injection. *C*: second CIA experiment (*design 2*) was a completely randomized design. Treatments were dietary CLA+CII-immunization; dietary CO+CII-immunization; and dietary CO+sham immunization. In *design 2* of the CIA model all animals were fed the CO diet (21 days) before the initial injection. After the booster injection, mice were either fed the CO or the CLA diet for the remaining 56 days of the study. Arthritis scores were determined every 2 or 3 days during the feeding period.

Table 1. Experimental diet contents for CLA and CO diets

Ingredient, g/100 g	Mouse Diet	
	CLA	CO
Sucrose	47.6	47.6
Casein	21	21
Corn starch	15	15
Corn oil	5.5	6
Cellulose	5	5
AIN-76 Mineral mix	3.5	3.5
AIN-76 Vitamin mix	1	1
Calcium carbonate	0.4	0.4
DL-Methionine	0.3	0.3
Choine bitartrate	0.2	0.2
Ethoxyquin	0.001	0.001
CLA90	0.5	

CLA, Conjugated linoleic acid; CO, corn oil.

following criteria: 0, normal, 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of ankle and wrist; 3, severe redness and swelling of the entire paw, including digits; 4, maximally inflamed limb with involvement of multiple joints. Each paw was examined, and the scores were combined for a total possible score of 16 per animal (48). On day 10, animals were euthanized, and right hind paws from randomly selected animals in each group were taken for histological analysis.

**CIA model.** Four-week-old male DBA/1 mice were purchased from Harlan (Indianapolis, IN) and acclimatized to the animal unit at least 3 days on a chow diet and under a 12:12-h light-dark cycle. After acclimation, mice were assigned randomly to their experimental treatments as shown in Fig. 1. Diets at this time consisted of the semipurified diet (see above under CAIA) with either CO or CLA.

In the first CIA experiment, mice were fed either CLA or CO ( $n = 8$  per diet) 3 wk before immunization with either CII or vehicle ( $2 \times 2$  factorial arrangement of treatments with diet and injection type as main effects,  $n = 4$  per group, see Fig. 1B). In the second experiment, all mice were fed the semipurified CO diet ( $n = 27$ ) until the time of the booster injection, at which time 9 mice were switched to the CLA diet (Fig. 1C), and the other 18 were maintained on the CO diet.

Immunization and data collection procedures were similar in both CIA experiments. On day 0, mice were given 100  $\mu$ l of the primary injection intradermally at the base of the tail. The primary injection consisted of either 100  $\mu$ g chick CII dissolved in 0.05 M acetic acid (Chondrex, Redmond, WA) or 0.05 M acetic acid (sham-injection) emulsified in a 1:1 ratio with complete Freund's adjuvant (2 mg/ml heat-killed *Mycobacterium tuberculosis*; Sigma, St. Louis, MO). On day 21, mice received 100  $\mu$ l of the booster injection intraperitoneally. The booster injection consisted of either 100  $\mu$ g chick CII dissolved in 0.05 M acetic acid or 0.05 M acetic acid (sham injections) emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Sigma). On days -7 and 35, blood was collected from the retroorbital venous plexus, while mice were under light isoflurane anesthesia. Plasma was separated and samples were stored at -80°C until analyzed. The clinical inflammation (arthritis) score was monitored three times per week as above.

**Plasma anti-CII antibody EIA.** Immunolon II HB plates (Fisher Scientific, Pittsburgh, PA) were incubated overnight with chick CII (100  $\mu$ g/ml; Chondrex). Plates were blocked with 1% BSA (Sigma) in PBS. Either test plasma samples or a known amount of anti-CII monoclonal antibody clone MAB8887 (Chemicon) were added to the plate in serial dilutions and incubated for 2 h. The plate was washed five times before a 2 h room temperature incubation of a 1:2,000 dilution of anti-mouse IgG antibody coupled to horseradish peroxidase enzyme (gamma chain specific; Sigma) in 1% BSA. Substrate

solution (0.05 M Na acetate, 3.2 mM H<sub>2</sub>O<sub>2</sub>, 0.1 mg/ml 3,3', 5,5'-tetramethyl benzidine, pH = 4.4) was added to the plate for 30 min. The color reaction was stopped by addition of 0.05 M H<sub>2</sub>SO<sub>4</sub>. The optical density was read at 450 nm with a reference wavelength of 600 nm on an ELx808 Ultra microplate reader (Bio-Tek Instruments, Winooski, VT). Anti-CII titer was calculated based on a standard curve (in the linear range) based on serial dilutions of anti-CII monoclonal antibody clone MAB8887. CII reactive IgG isotypes were detected by the same method using biotinylated anti-mouse IgG<sub>1</sub> or IgG<sub>2a</sub> antibodies with streptavidin conjugated horseradish peroxidase enzyme (BD Pharmingen, San Jose, CA) in the place of the gamma chain specific anti-mouse IgG detection antibody.

**TNF ELISA.** Opti-EIA murine TNF ELISA kit (BD Pharmingen, San Jose, CA) was used to measure plasma TNF concentration according to the manufacturer's instructions.

**Histology.** At the conclusion of the CAIA study, right hind paws were removed from randomly selected animals in each experimental group. The paw sample included all of the digital bones and the ankle joint. Paw samples were fixed in 10% buffered formalin for 1 wk before decalcification in Cal-EX solution (Fischer Scientific, Pittsburgh, PA) for 24 h. Samples were stored in 10% buffered formalin. Samples were embedded in paraffin and sectioned. Slides were stained with hematoxylin and eosin stains, and slides were photographed under  $\times 4$  magnification.

**Statistics.** A moving average of clinical arthritis scores was determined by calculating the mean of two consecutive observations for a given paw at each time point (CIA experiment 1 only). Arthritis score data were analyzed using the SAS program (SAS Institute, Cary, NC) with the mixed procedure accounting for autocorrelation of repeated measures. Antibody titers and TNF concentrations were analyzed using ANOVA. Results were considered significant with a  $P$  value  $< 0.05$  unless otherwise stated.

## RESULTS

**CAIA model.** Inflammation in the CAIA model began developing within 24 h after the LPS injection. At the peak of inflammation, occurring on days 4-6, arthritis scores in the CO-fed antibody-injected group scored near 6 (Fig. 2). A statistically significant reduction in inflammation in the CLA-fed antibody-injected group occurred on days 5 and 6 with arthritis scores 60% of CO fed antibody-injected group scores (Fig. 2). Figure 3 illustrates joint histology of right-rear hind paws of randomly selected mice in each group. The ankle joint of the mAb-CO animal (Fig. 3B) shows significant cell infiltration and joint remodeling compared with the same joint in the sham-CO animal (Fig. 3A). The ankle joint from the mAb-CLA animal (Fig. 3C) shows a well-structured ankle

Table 2. Fatty acid contents of dietary oils

Fatty Acid	CLA	CO
c16:0	0.6	12.8
c18:0	n/d	2.4
c18:1 c9	6.4	29.1
c18:1 c11	0.3	0.8
c18:2 c9, c12	0.3	53.2
c18:2 c9, t11	42.7	n/d
c18:2 t10, c12	43.5	n/d
c18:2 cc*	1.9	n/d
c18:2 tt†	4.1	n/d
c18:3 c9, c12, c15	n/d	1.2
c20:0	n/d	0.5

Analysis of test oils by gas chromatography test oil. \*Conjugated *cis*, *cis* isomers; †conjugated *trans*, *trans* isomers; n/d, not detectible.

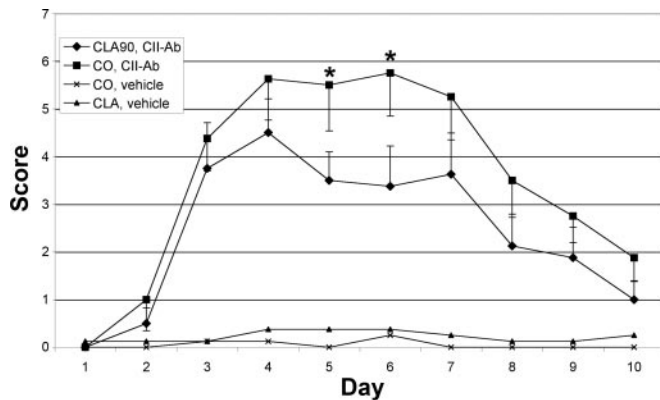


Fig. 2. Mean arthritis score after feeding CLA or CO in the CAIA model. Diamonds represent CLA fed mice immunized against CII, and squares represent immunized CO fed mice. Triangles and crosses represent sham-immunized mice fed CLA or CO, respectively. After 3 wk of diet feeding mice were injected intravenously with 2 mg of a cocktail of four anti-CII monoclonal antibody clones. After 24 h, mice were injected intraperitoneally with 50  $\mu$ g of LPS in sterile PBS. Clinical arthritis score was monitored, as described in the MATERIALS AND METHODS, for 10 days. Scores were based on the following criteria: 0, unaffected; 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of ankle and wrist; 3, severe redness and swelling of the entire paw including digits; 4, maximally inflamed limb with involvement of multiple joints. Data were analyzed using SAS Proc Mixed accounting for autocorrelation due to repeated measures. \*Significant differences between antibody-injected CO- and CLA-fed groups with  $P < 0.05$ .

joint. TNF was measured in blood drawn 1 h after injection of LPS. The CO-fed, LPS-injected mice had plasma TNF concentrations of 700 pg/ml, whereas the TNF concentration in CLA-fed mice was 389 pg/ml ( $P = 0.03$ ). Plasma TNF levels confirmed that the CLA had a biological immune effect in this study (58).

**CIA model.** In the first CIA experiment, mice were fed CLA or CO diets beginning 21 days before the immunization protocol (Fig. 1B). On *day 0*, one-half of the mice from each diet group were immunized against chick CII and the other half of the mice were sham-immunized ( $2 \times 2$  factorial design;  $n = 4$  per group). Clinical signs of arthritis were not apparent before the booster injection in the CO-fed mice; however, the CLA-fed mice exhibited signs of inflammation before *day 21*, booster injection of CII. Apart from mild inflammation of the injection site typically associated with Freund's complete adjuvant with or without the vehicle, sham-immunized mice did

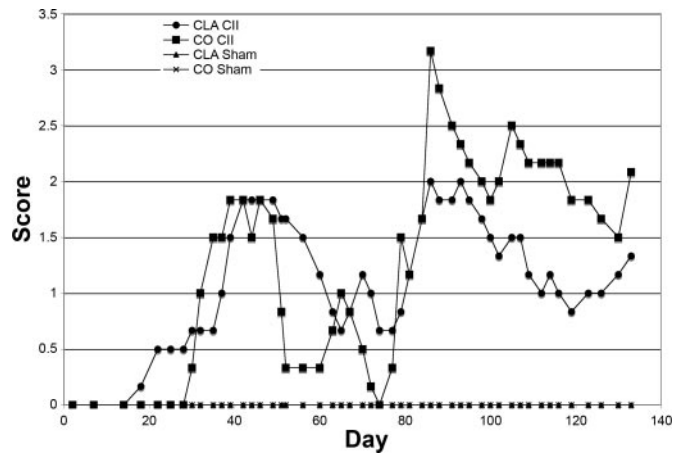


Fig. 4. Two-point moving average of arthritis score over time in mice, fed CLA or CO for 21 days before induction of arthritis, in the CIA model. Circles represent CLA-fed mice immunized against CII, and squares represent immunized CO-fed mice. Triangles and crosses represent sham-immunized mice fed CLA or CO, respectively. Mice ( $n = 4$  per group) were immunized by intradermal injection of with an emulsion of chick type II collagen in an equal volume of Freund's complete adjuvant at the base of the tail on *day zero*. On *day 21*, mice were given an intraperitoneal booster injection of an emulsion of chick CII in incomplete Freund's adjuvant. (See MATERIALS AND METHODS for details on diets and injections) Clinical arthritis score was monitored throughout the experiment. Each paw was scored based on the following criteria: 0, unaffected; 1, mild, but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of ankle and wrist; 3, severe redness and swelling of the entire paw including digits; 4, maximally inflamed limb with involvement of multiple joints. The scores for all four paws were summed for a total possible score of 16 per animal.

not exhibit signs of arthritis at any time regardless of diet. Arthritis score became apparent in the CO-fed mice only after the booster injection with CII, and scores increased in both CLA- and CO-fed mice until peaking at a score of 2 to 3 from *day 30* to *day 49* (Fig. 4). Apart from the early appearance of inflammation signs in the CLA-fed mice, there was no overall significant difference in arthritis score between CII-immunized mice fed CLA, and mice continued to be fed their dietary treatments and scored for inflammation until *day 133* post-CII injection; however, no additional sampling was done during this period. Beginning on *day 80* and to the end of the observation period, a relapse of joint inflammation occurred. Mice in the CLA-fed group had a 30% reduction in arthritis scores compared with the CO-fed group (differences were nonsignificant).

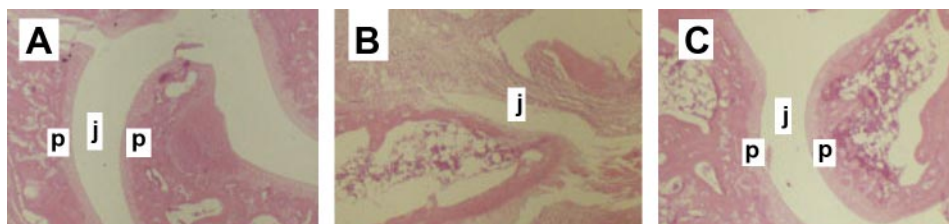


Fig. 3. Ankle joint histology from right hind paw of anticollagen antibody-induced arthritis mice. Mice were injected with monoclonal antibodies against type II collagen to induce arthritis (see MATERIALS AND METHODS for details). At the conclusion of the study, the right hind paws were removed. Paw samples were fixed in 10% buffered formalin for 1-wk before decalcification in Cal-EX solution for 24 h. Randomly selected samples were embedded in paraffin and sectioned. Slides were stained with hematoxylin & eosin stains. A: corn oil-fed sham-injected. B: corn oil-fed antibody injected. C: CLA-fed antibody injected. p, epiphyseal plate; j, joint space. Sections from the sham-injected mouse appear normal (A), while sections from the CO-fed antibody injected mouse exhibit infiltration of cells and erosion of the epiphyseal plate (B). Sections from the CLA-fed antibody-injected mouse appear similar to the sham-injected sections (C). Magnification shown is  $\times 4$ .

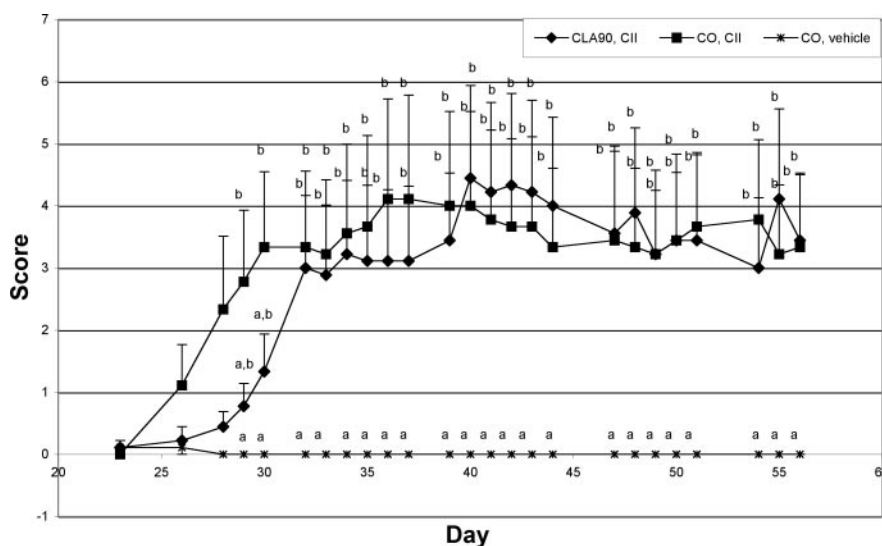


Fig. 5. Mean arthritis score over time in mice fed CLA or CO after immunization against CII in the CIA model. Diamonds represent mice immunized against CII fed CLA, and squares represent immunized CO-fed mice. Crosses represent sham-immunized mice fed CO. Mice ( $n = 9$  per group) were immunized at the base of the tail with an emulsion of chick type II collagen in an equal volume of Freund's complete adjuvant on *day zero*. On *day 21*, mice were given a booster injection of an emulsion of chick CII in incomplete Freund's adjuvant. (See MATERIALS AND METHODS for details on diets and injections.) Clinical arthritis score was monitored throughout the experiment. Each paw was scored based on the following criteria: 0, unaffected; 2, moderate redness and swelling of ankle and wrist; 3, severe redness and swelling of the entire paw including digits; 4, maximally inflamed limb with involvement of multiple joints. The scores for all four paws were summed for a total possible score of 16 per animal. SAS Proc Mixed was used to analyze data. Letters represent significant differences with  $P < 0.05$ .

In the second CIA experiment, mice were maintained on a CO diet prior to and up to the 21-day CII booster injection. At the time of booster injection, one-third of the mice ( $n = 9$ ) were switched to a CLA diet, while the other mice ( $n = 18$ ) were maintained on a CO diet (Fig. 1C). The CLA mice and one-half of the CO mice were immunized against chick CII, while one-half of the CO fed mice were sham-immunized to complete a three treatment completely randomized design ( $n = 9$  per group). As observed in the first CIA experiment, CO-fed mice began showing signs of inflammation within about 5 days after the booster injection of CII. CLA-fed mice showed an ~4-day delay in reaching arthritic scores of 1, 2, 3, and 4. Apart from the delayed signs of inflammation in the CLA-fed mice, the arthritis score increased after the booster injection in both CII-immunized CLA- and CO-fed mice in a similar manner and reached a plateau (score of ~4) until *day 56* (Fig. 5). Sham-immunized mice did not exhibit signs of arthritis at any time. CO-fed CII-immunized mice were statistically different from the sham group by *day 28*, while CLA-fed mice were not statistically different from the sham-immunized mice until *day 33*.

Plasma total anti-CII IgG, anti-CII IgG<sub>1</sub>, and anti-CII IgG<sub>2a</sub> titers were measured in both CIA experiments. CII-specific antibody was not detected in plasma prior to immunization (*day -7*, data not shown) or in sham-injected groups from samples taken on *day 35* in both experiments. In the first CIA experiment, there was a reduction in antigen-specific total anti-CII IgG and anti-CII IgG<sub>1</sub> titer in the CLA-fed and CII-immunized mice compared with the CO-fed and CII-immunized mice ( $P = 0.06$  and  $P = 0.08$ , respectively; Table 3). There was no difference in anti-CII IgG<sub>2a</sub> titer between CLA- and CO-fed and CII immunized mice. (However, it may be important to note that there was a 1.4-fold increase in IgG<sub>2a</sub> as a result of CLA). In the second CIA experiment, total anti-CII

IgG (CLA = 1,403 vs. CO = 3,506) and anti-CII IgG<sub>1</sub> (CLA = 784 vs. CO = 979) were not statistically different, even though the number of mice was increased, and while anti-CII IgG<sub>2a</sub> (CLA = 3,676 vs. CO = 2,413) titers were not affected by diet, a 1.5-fold increase was evident as in the first CIA experiment (see DISCUSSION).

## DISCUSSION

To study the effects of CLA on inflammation in RA, two models were used: CAIA and CIA. The CAIA model of RA is solely dependent on antibody-initiated inflammation of joints, whereas the CIA model is dependent on both the acquired immune response to the injected CII antigen and the resulting antibody. Hence, use of these two models of arthritis allowed for differentiation between CLA's effects on antibody and acquired immunity-mediated events in joint inflammation (35). Mice fed CLA for 3 wk before application of CAIA methods showed a significant decrease in joint inflammation. COX-2 null mice showed reduced inflammation when CAIA was applied (33). Because dietary CLA has been shown to decrease COX-2 expression, and resulting eicosanoids in response to both reoccurring antigen exposure (51, 52) and endotoxin (24),

Table 3. Anti-type II collagen IgG reactive titer on *day 35* of the first CIA experiment

	Collagen Immunized		Sham Injected		Main Effect		
	CLA	CO	CLA	CO	Diet	Injection	Interaction
Total IgG	1277 (82)	458 (32)	n/d	n/d	0.0627	<0.0001	0.0627
IgG1	434 (188)	1290 (400)	n/d	n/d	0.0771	0.0021	0.0771
IgG2a	669 (278)	485 (103)	n/d	n/d	0.5474	0.0022	0.5474

Values are expressed as means  $\pm$  SE.

a possible mechanism of CLA-induced suppression of inflammation associated with CAIA may have involved COX down-regulation. In addition, dietary CLA has also been shown to reduce TNF in response to LPS [shown here and elsewhere (4, 5, 17, 56)]. TNF is an integral proinflammatory cytokine in CAIA (20). Inhibition of TNF through the use of anti-TNF antibodies, soluble TNF receptors, or antagonist of TNF-converting enzyme (TACE) have been used as therapeutic strategies for RA (36, 37, 54). The CLA-induced reduction in TNF, found 1 h after LPS injection in the CAIA model, may be relevant to the anti-inflammatory effects observed days later. Newton et al. (37) showed that inhibition of TACE-dependent release of soluble TNF in the CAIA model (TNF released independent of downstream gene transcription and translation) reduced the cascade of cytokines released subsequent to TNF release and reduced clinical scores of inflammation in the 11 days following the LPS activation of the disease. Coppieters et al. (10) showed the use of camelid antibodies to TNF resulted in decreased inflammation of joints in the murine model of CIA similar to what was shown in this study (Fig. 3). The use of anti-TNF or anti-IL-1 antibodies before the onset of CIA was also effective at delaying the onset of arthritis but was ineffective at preventing the clinical signs of arthritis (54). Hence, dietary CLA's effects on TNF should also be considered as a possible mechanism by which CLA reduced CAIA. CLA-induced suppression of antibody-dependent inflammatory responses have also been demonstrated in the airway (51, 52), where antigen-induced contraction of trachea (% of maximum contraction) was decreased when harvested from guinea pigs fed CLA. Anaphylaxis in response to egg white lysozyme was decreased by dietary CLA in mice (19). Longevity of NZB/W F1 mice that spontaneously develop lupus erythematosus was extended 1.5-fold if the mice were fed CLA compared with corn oil (55). Hence, anti-inflammatory effects of dietary CLA in the antibody-dependent model of CAIA were anticipated and consistent with previous reports in other antibody-dependent disease models.

A second study was conducted using the same feeding strategy described for CAIA to investigate CLA's effects during an operative acquired immune response. Mice were subjected to the CIA protocol 3 wk after being fed a CO- or CLA-supplemented diet. We hypothesized that the inflammatory response in the CIA model would be different from the CAIA model because the adaptive immune response was engaged in CIA (1). This anticipated difference was based on previous work that had shown that dietary CLA has a broad array of effects on acquired immune responses (7, 8, 16, 40). Recent work has also suggested that dietary CLA may shift an acquired immune response toward a Th-1 type reaction (7, 56). Evidence supporting a shift toward a Th-1 reaction was decreased IgE antibody production in lymphocytes from rats fed CLA (45) and decreased IL-4 and increased IL-2 in concanavalin-A stimulated splenocyte harvested from mice fed CLA (56). RA and CIA are well recognized as Th-1 diseases (9, 13, 28, 29). Our findings in the CIA experiment of an effect of dietary CLA on IgG<sub>1</sub> (66% decreased) and IgG<sub>2a</sub> (1.4-fold increase) were supportive of a shift toward a Th-1 response. Cooper et al. (9) showed that a change in IgG<sub>2a</sub> anti-CII levels (1.6-fold difference) was associated with the incidence of CIA disease (increased anti-CII IgG<sub>2a</sub> was associated with more severe disease). In another study, decreasing anti-CII IgG<sub>2a</sub> by

binding TNF, with soluble TNF receptor, reduced lesion scores in the CIA model (32). Hence, previous reports suggesting that CLA may select for a Th-1 response and the antibody profiles reported here would be supportive of increased clinical signs of CIA in CLA-fed mice. Indeed, mice fed CLA showed their first clinical signs of disease 10 days before control fed mice. In another Th-1 disease, lupus erythematosus, NZB/W F1 mice fed CLA developed antinuclear antibodies and proteinuria earlier than control fed mice (57). Hence, the earlier clinical signs in both the CIA (here) and lupus (57) models of inflammation supported a CLA-induced shift toward a Th-1 type response. Peak inflammation in the CIA model was not affected by diet (power analysis was sufficient to detect the 40% change observed in CAIA). Although no differences in long-term clinical signs of arthritis were detected, it is possible that long-term changes in joint structure could have occurred had histological analysis of joint tissue been examined (not done). An earlier appearance of anti-nuclear antibodies and proteinuria in CLA-fed NZB/W F1 mice was not associated with a decreased life span, as would be predicted based on earlier appearance of disease signs (57). It was possible that the antibody-dependent anti-inflammatory effects observed in the CAIA model may have counteracted the enhanced disease-causing effects of a shift toward Th-1 responses in mice fed CLA in a manner similar to what was found in the lupus model (56).

The CIA study described above was continued (133 days) longer than typically reported using this model (a length of less than 70 days is typical in CIA studies). The extended study of the CIA mice afforded an opportunity to observe the effects of dietary CLA during a "relapse" of joint inflammation. By *day* 70, the mean arthritis scores for both control- and CLA-fed mice had declined below a score of 1 [a pattern consistent with Myers et al. (33)]. Beginning ~80 days after the first CII-injection, an unexpected relapse of joint inflammation was observed, a response that has not been well studied (1, 46); hence, antibody titers were not measured. During the relapse, joint swelling in the CLA-fed mice was reduced ~30% (not statistically significant possibly because the study was powered to detect a 40% reduction in arthritis scores). Because no serological analysis was done at this time, any comment on these data would be speculative. It has been reported that DBA/1 mice may develop arthritis spontaneously (11, 39). The possible effect of dietary CLA during the relapse period could be similar to what has been reported in the lupus model of autoimmunity. In a study involving spontaneous lupus, NZB/W F1 mice had increased longevity (1.5-fold) and reduced end-stage body weight wasting following proteinuria if fed CLA (55, 57). Hence, it is possible that an anti-inflammatory response associated with feeding CLA during the relapse period was antibody dependent and similar to what was observed in the CAIA model described above and the lupus model described previously (57).

In the third experiment, an attempt was made to test the hypothesis that CLA may be protective against CIA if the CLA were fed after the first injection of CII (feeding commences with the second or booster injection). The results of this experiment showed a delay (~4 days) in the appearance of clinical signs of joint swelling in the CLA-fed mice compared with the control-fed mice, but peak scores were similar for mice fed the two diets. The IgG<sub>2a</sub> was increased 1.5-fold

(nonsignificant) in the CLA-fed mice compared with the control-fed mice, suggesting that feeding CLA at the booster injection may have remained too early to test the intended hypothesis. However, these data were supportive of the first CIA experimental findings that CLA did not exacerbate the clinical signs of arthritis.

Commercially prepared mixed isomers of CLA (predominantly c9, t11, and t10, c12-CLA) were used in the experiments presented. Currently, dietary supplements of mixed isomers with the two major isomers shown are the only CLA supplements available to the general public. Research has shown that the biological activity of the individual isomers may yield results very different than the pure isomers. For example, the c9, t11 CLA isomer was the isomer reported to inhibit the release of TNF in LPS-injected mice (t10, c12 had minimal effects) (56), whereas, the t10, c12 isomer appeared to be the isomer most effective in the regulation of COX-2 expression (24). Butz et al. (4) showed that the mixed isomers of CLA were necessary for the prevention of LPS-induced weight loss (neither isomer alone was effective); however, when mice were fed the t10, c12 isomer following LPS injection, gain following weight loss was compensatory. It is anticipated that the response of mice to individual isomers may be different than what is presented in this study. However, since each isomer alone (c9, t11-CLA affects TNF and t10, c12-CLA affects COX-2) has an effect on inflammatory mediators involved in arthritis (24, 56), it was decided that a study of commercially available sources of CLA should be studied and reported.

In conclusion, the results of these experiments suggested that dietary CLA might have had a moderate ability to prevent CII antibody-dependent joint inflammation. CLA's anti-inflammatory affect may have been less evident if fed during the development of an acquired immune response to CII than after the acquired immune event was complete. The diminished anti-inflammatory affect of dietary CLA during the acquired immune response may have been due to CLA's tendency to drive a Th-1 type of immune response. The antibody-dependent anti-inflammatory effects of CLA may have counteracted CLA's possible drive toward a Th-1 immune response, which, in turn, may have explained why arthritis scores were not increased when CLA was fed prior to CIA.

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