ACADEMIC PRESS

Pharmacological Research 47 (2003) 43–47

Pharmacological research

www.elsevier.com/locate/yphrs

Synthesis of cetyl myristoleate and evaluation of its therapeutic efficacy in a murine model of collagen-induced arthritis

Kenneth W. Hunter, Jr.[a,*], Ruth A. Gault[a], Jeffrey S. Stehouwer[b], Suk-Wah Tam-Chang[b]

[a] *Department of Microbiology, University of Nevada School of Medicine, Reno, NV 89557, USA*
[b] *Department of Chemistry, University of Nevada, Reno, NV 89557, USA*

Accepted 3 September 2002

Abstract

Cetyl myristoleate (CM) was reported by Diehl and May [J Pharm Sci 83 (1994) 296] to block inflammation and prevent adjuvant-induced arthritis in rats. To verify this earlier work, we have synthesized pure CM and tested its anti-arthritic properties in a collagen-induced arthritis model in DBA/1LacJ mice. Multiple intraperitoneal injections of CM in 450 and 900 mg kg^{-1} doses resulted in a significantly lower incidence of disease and caused a modest but significant diminution in clinical signs in those mice that developed arthritis. CM administered in daily oral doses of 20 mg kg^{-1} also reduced the incidence of arthritis and caused a small reduction in the clinical signs in mice that developed arthritis. Although the protective effect of CM in collagen-induced arthritis observed in the present study was less dramatic than that reported earlier, our results confirm the anti-arthritic properties of pure CM.

Keywords: Cetyl myristoleate; Synthesis; Collagen-induced arthritis; Inflammation

1. Introduction

Cetyl myristoleate (CM) is the ester of *cis*-9-tetradecenoic acid (myristoleic acid) and 1-hexadecanol (cetyl alcohol). CM was originally isolated as a natural product from a NIH Swiss albino mouse strain that was resistant to adjuvant-induced arthritis [1]. When tested in a rat adjuvant arthritis model, this compound was shown to have striking anti-arthritic properties, albeit when used in very high doses. These workers later synthesized CM and demonstrated a similar protective effect. Nutraceuticals containing CM are widely used for indications of pain and inflammation relief, and with the exception of a short report suggesting a positive clinical effect of cerasomol-CM in patients with fibromyalgia [2], no follow-on studies have been published to confirm any biological properties of this fatty acid ester. Moreover, because over-the-counter preparations of CM contain free fatty acids that have been shown to modulate inflammation in arthritis and other inflammatory diseases [3], it was important to confirm that pure CM has anti-arthritic properties. Therefore, in this study we have synthesized pure CM and tested its anti-arthritic properties in a mouse collagen-induced arthritis model. We report here that pure CM in doses of 450 and 900 mg kg^{-1} given intraperitoneally (i.p.) caused a significant reduction in both the incidence and severity of arthritis in mice. In addition, CM given orally in doses of 20 mg kg^{-1} significantly reduced the incidence and caused a modest reduction in the severity of arthritis.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female DBA/1LacJ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The animal protocol was approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

2.2. Collagen-induced arthritis model

In the first study, each of 30 female DBA/1LacJ mice was injected intradermally at two sites on their shaved backs with a total of 100 mg of bovine type II collagen (Chondrex, Redmond, WA, USA) emulsified in complete Freund's adjuvant (CFA, Difco, Detroit, MI, USA). The

* Corresponding author. Tel.: +1-775-784-1620; fax: +1-775-784-6161.
E-mail address: khunter@unr.edu (K.W. Hunter Jr.).

1043-6618/02/$ – see front matter © 2002 Published by Elsevier Science Ltd.
PII: S1043-6618(02)00239-6

mice were randomized into three groups of 10 mice, and on day 12 each group was injected via the i.p. route with 100 μl of either phosphate buffered saline (PBS, vehicle control), 450 mg kg^{-1} CM, or 900 mg kg^{-1} CM. The PBS or CM injections were repeated every third day until day 30 post arthritis induction (seven total doses). Note that the dosages of CM were chosen based on those used in the original study by Diehl and May [1]. It should also be noted that CM is very insoluble in PBS, so the correct dose was added as a layer on 100 μl of PBS, pulled into a 1 cm^3 tuberculin syringe, and injected as a "suspension" of CM in PBS. The mice were observed daily for clinical signs of arthritis.

In the second study, 20 mg kg^{-1} of CM was given per os (p.o.) to the mice on a daily basis throughout the course of the experiment. The CM was adsorbed onto a 1 mg food pellet (P.J. Noyes Company, Lancaster, NH, USA), and each animal was fed one CM-treated or control pellet each day prior to ad libitum feeding of normal mouse chow. Mice were fasted from 19:00 to 07:00 h to facilitate consumption of the food pellet. The study consisted of 31 female DBA/1LacJ mice, 28 of which were injected with bovine type II collagen as described above. In a variation from the procedure in the first study, the mice were given a booster immunization with type II collagen in Freund's incomplete adjuvant on day 28. Fourteen mice were given the daily p.o. CM treatment, while 14 were given placebo (a food pellet without CM). Three mice were given the CM treatment, but were not immunized with type II collagen. Mice were observed daily for signs of arthritis just as described earlier, and each mouse was weighed daily.

2.3. Statistical analysis

The percentage of mice showing any signs of arthritis was determined for control and CM treatment groups, and a binomial test used to determine the significance of the difference in incidence of disease [4]. The severity of arthritis was evaluated on a 5-point clinical scale (Table 1), and the significance of differences between the means of the control mice compared to each CM treatment group on the same day post-arthritis induction was determined by Student's *t*-test. On the 5-point clinical scale, the highest severity level attained by each mouse regardless of day was determined, and the means of these maximum severity scores for control and CM-treated groups were compared by Student's *t*-test.

Table 1
Clinical scale for assessing development of arthritis in mice

1	No evidence of erythema or edema
2	Erythema and mild edema confined to midfoot (tarsals) or ankle joint, or front paw and wrist (carpals)
3	Erythema and edema extending from the ankle joint to the tarsals, or from the elbow joint to the carpals
4	Erythema and moderate edema from the ankle to the metatarsal joints, or from the elbow to the metacarpal joints
5	Significant erythema and edema of all major joints of at least one limb

2.4. Histopathology

Mice were sacrificed with halothane, and then a portion of their hind limb extending 2 mm proximal and distal to the ankle joint was excised and placed in 10% neutral buffered formalin. After fixation, the bone was decalcified using Decal-Stat (American Master*Tech, Lodi, CA, USA), and then the tissues were processed in an automated tissue processor with final embedding in paraffin. Sections were cut with a microtome at 7.5 μm, mounted on slides, and then stained with standard haemotoxylin and eosin (H&E). Thin sections were examined with a Nikon Photoscope under bright field illumination, and photographs of sections were made with a Kodak digital camera.

2.5. Synthesis and analysis of cetyl myristoleate

Since pure CM is not commercially available, it was synthesized by the method described below. Myristoleic acid (*cis*-9-tetradecenoic acid) and cetyl alcohol (1-hexadecanol) were purchased from Aldrich, Milwaukee, WI, USA. Flash silica gel #02826-5, 32–63 μm particle size, 60 Å pore size was obtained from Scientific Adsorbents, Inc., Atlanta, GA, USA, and TLC Plates #5719-2 silica gel 60 F_{254}, 250 μm thickness were obtained from EM Sciences, Agawam, MA, USA. Visualization was done with cerium sulfate/ammonium molybdate in $H_2SO_{4(aq)}$ (10% v/v). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained on a Varian Unity Plus NMR spectrometer at 500 and 125 MHz, respectively, and are referenced to internal TMS. Infrared (IR) spectra were obtained on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. High Resolution Mass Spectrometry (HRMS) was performed by the University of California, Riverside Mass Spectrometry facility.

Myristoleic acid (1.11 g, 4.91 mmol), cetyl alcohol (2.57 g, 10.62 mmol, 2.2 equivalents), *p*-toluene sulfonic acid monohydrate (0.52 g, 2.75 mmol, 0.6 equivalent), and benzene (200 ml) were heated at reflux under $N_{2(g)}$ for 22 h with azeotropic removal of water. The solvent was removed to give an off-white solid that was vacuum flash chromatographed on silica (9 cm h × 4 cm w, $CHCl_3$, TLC R_f = 0.58) to afford 2.01 g (91%) of a clear oil: HRMS (DEI) *m/z* calcd for $C_{30}H_{58}O_2$ (M^+): 450.4437; found: 450.4454; IR (neat/NaCl) 2924, 1740, 1655, 721 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) 5.36–5.33 (m, 2H, J = 3 Hz), 4.05 (t, 2H, J = 6.8 Hz), 2.29 (t, 2H, J = 7.8 Hz), 2.01 (m, 4H, J = 3 Hz), 1.61 (m, 4H, J = 6.8 Hz), 1.30–1.26 (br, d, J = 38 Hz), 0.89 (q, 6H, J = 7.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) 174.16, 130.13, 129.97, 64.61, 34.62, 32.18, 32.15, 29.92, 29.91, 29.90, 29.88, 29.87, 29.80, 29.75, 29.58, 29.48, 29.38, 29.35, 29.32, 28.88, 27.37, 27.13, 26.16, 25.23, 22.91, 22.56, 14.32, 14.20.

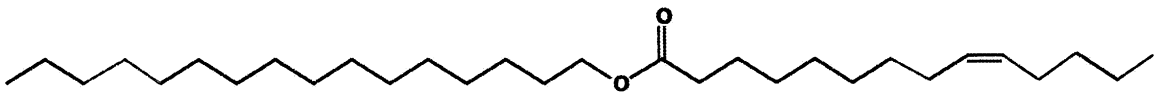

Fig. 1. Structure of cetyl myristoleate ($C_{30}H_{58}O_2$; MW, 450.7803; C = 79.93%, H = 12.97%, O = 7.10%).

3. Results

CM was successfully synthesized from *cis*-9-tetradecenoic acid and 1-hexadecanol using a modification of the original method described by Diehl and May [1]. The end product was a clear oily liquid at room temperature. Using NMR, IR, and HRMS analysis methods, the synthetic procedure described yielded a product consistent with the known structure of cetyl myristoleate ($C_{30}H_{58}O_2$; MW, 450.7803; see Fig. 1).

In the first experiment male DBA/1LacJ mice were given i.p. injections of CM beginning on day 12 following induction of arthritis and continuing every third day until day 53. As can be seen in Table 2, 7 of 10 control animals (70%) showed signs of arthritis, whereas only 3 of 10 (30%) and 4 of 10 (40%) mice developed signs of arthritis in the 450 and 900 mg kg^{-1} CM-treated groups, respectively. Table 3 shows that clinical signs of arthritis tended to develop more rapidly in the PBS control than in the CM-treated groups, and the severity scores were significantly different by day 57. Moreover, the maximum severity scores of both CM-treated groups were significantly different from the PBS controls.

In the second experiment, mice were given daily oral doses of a saline-treated food pellet or 20 mg kg^{-1} CM. In the placebo group, 65% of the mice showed some signs of arthritis (independent of severity), whereas only 36% of the CM-treated mice showed any signs of arthritis. Beginning on day 32, and more prominently on days 36 and 42, the placebo-treated control mice had higher average severity scores than the mice given daily oral doses of CM. Although the differences between the means of these severity scores did not achieve significance at the usual 95% confidence level, the day 42 results were significant at the 90% confidence level (one-tailed *t*-test). In addition, when the maximum severity score for each mouse was determined, there was a difference between the CM-treated mice and placebo controls significant at $P = 0.13$. It should also be pointed out that two of the placebo mice showed signs of systemic inflammatory disease and died on days 43 and 44, respectively. No CM-treated mice showed any signs of systemic inflammatory disease. The food deprivation regimen did not cause a significant decrease in the weights of the mice when compared to ad libitum fed controls (data not shown).

Gross examination of affected limbs revealed significant erythema and edema characteristic of inflammatory joint disease. Fig. 2 compares the normal histology of the mouse ankle joint with the histopathology seen in an animal with collagen-induced inflammatory joint disease (severity score 5). It should be noted that the normal joint space is lined with a thin layer of synovium overlaying the cartilaginous layer. Deep to the cartilage is hard bone, which in these decalcified sections, appears as an eosinophilic matrix. The synovium is relatively acellular and can be seen at the corner of the joint space. In contrast to the normal histology of the mouse joint, the arthritic mouse joint shows characteristic signs of inflammation. In this example, the normal architecture of the cartilaginous layer has been disrupted with a

Table 2
Effect of CM on the incidence of collagen-induced arthritis in DBA/1LacJ mice

Group	Incidence[a]	Statistical analysis[b]
First study		
PBS i.p. control	70% (7/10 mice)	
450 mg kg^{-1} i.p. CM	30% (3/10 mice)	vs. PBS control, $P = 0.02$
900 mg kg^{-1} i.p. CM	40% (4/10 mice)	vs. PBS control, $P = 0.04$
Second study		
Placebo control	65% (9/14 mice)	
20 mg kg^{-1} CM orally	36% (5/14 mice)	vs. Placebo $P = 0.02$

[a] Number of mice that develop any clinical signs of arthritis/total number of mice.
[b] Binomial test [13].

Table 3
Effect of CM on clinical severity scores in DBA/1LacJ mice with collagen-induced arthritis

Day	Severity scores (mean ± S.D.)		
	PBS i.p.	450 mg kg^{-1} i.p. CM	900 mg kg^{-1} i.p. CM
First study			
28	1.3 ± 0.5	1.1 ± 0.3	1.0 ± 0.0
36	1.4 ± 0.7	1.2 ± 0.4	1.3 ± 0.2
43	2.4 ± 1.7	1.4 ± 0.9[a]	1.6 ± 0.8
57	2.6 ± 1.8	1.4 ± 1.0[a]	1.2 ± 0.8[a]
Maximum[b]	3.0 ± 1.5	1.5 ± 1.0[a]	1.6 ± 0.8[a]
	Placebo p.o.	20 mg kg^{-1} p.o.	
Second study			
28	1.0 ± 0.0	1.0 ± 0.0	
32	1.9 ± 0.3	1.1 ± 0.3	
35	1.5 ± 0.5	1.3 ± 0.5	
38	1.4 ± 0.7	1.3 ± 0.5	
42	2.0 ± 1.0	1.4 ± 0.7	
46	1.7 ± 0.7	1.5 ± 0.8	
49	1.2 ± 0.4	1.2 ± 0.4	
Maximum[b]	2.1 ± 1.1	1.5 ± 0.8	

Post-immunization with collagen in CFA.
[a] $P < 0.05$ vs. PBS control (Student's *t*-test).
[b] Maximum severity score observed during course of experiment.

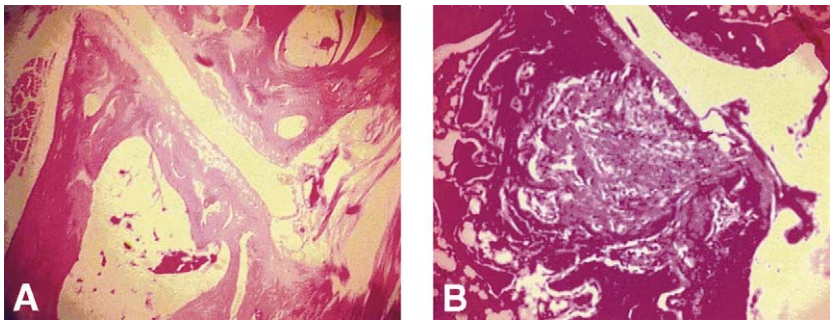

Fig. 2. Example of the histopathology of type II collagen-induced arthritis in the DBA/1LacJ mouse. H&E stain of decalcified sections of the ankle joints from mice on day 45 following immunization with collagen. (A) Mouse treated with 450 mg kg^{-1} CM as described in Section 2; (B) control mouse given saline on same regimen. Note the profound inflammatory changes and erosion seen in the joint of the control mouse.

locus of intense inflammation, characterized by the presence of macrophages, neutrophils, and fibroblasts. The inflammation has caused a near total erosion of the cartilaginous layer of the joint, and the erosion has extended into the bone and approaches the marrow cavity. In addition, there is evidence of an intense inflammatory synovitis. The histopathology seen in the mouse collagen-induced arthritis model is consistent with that seen in both human rheumatoid arthritis and osteoarthritis.

4. Discussion

The only paper describing an effect of CM on arthritis was a short report published by Diehl and May [1]. These authors isolated CM from the arthritis-resistant NIH Swiss albino mouse, and they also reported its isolation from "wild" mice. The only other report of CM as a natural product was in an abstract that identified CM in a mixture of waxes in the anal gland of male beavers, *Castor fiber* (L.) (cited from [1], Groenneberg TO, Chem Abstr 91:190204u). Diehl and May also synthesized the molecule using a method similar to that used in the present study. They used a rat adjuvant arthritis model and demonstrated that very high doses of CM administered parenterally significantly reduced the severity of arthritis. In contrast, in our first study we found more modest though statistically significant anti-arthritic effects in the mouse collagen-induced arthritis model using similar doses of CM delivered intraperitoneally. The differences noted might be explained by the differences in models, although both animal species serve as good models of rheumatoid arthritis in humans. The susceptibility of mice to collagen-induced arthritis is strongly linked to the major histocompatibility complex (MHC), with mice of the H-2^q and H-2^r haplotypes being most susceptible [5,6]. The H-2^q haplotype DBA/1LacJ that we employed is the most commonly used mouse strain for these studies. Immunization of genetically susceptible mice with bovine type II collagen in adjuvant induces the production of cross-reacting anti-collagen antibodies that bind to murine type II collagen in the synovia of the joints, and such binding precipitates a local inflammatory response characterized by macrophage activation, release of proinflammatory cytokines, neutrophil and macrophage-mediated destruction of the synovia, and eventually erosion of the cartilage and bone [7,8]. Adjuvant-induced arthritis, on the other hand, does not rely on the specific induction of anti-collagen antibodies by immunization, and thus is not MHC restricted [9]. Rather, adjuvant-induced arthritis manifests as a more generalized inflammation localized to the joint spaces, although the histologic picture is similar in both forms of arthritis [10,11].

As was already mentioned, the parenteral doses used in the Diehl and May study were exceptionally high. Nevertheless, we tested these same doses in our collagen-induced arthritis system to verify an anti-arthritic effect. Having demonstrated an effect, we chose to evaluate CM in a dose and route more in line with those provided in nutraceuticals and for which there is anecdotal evidence of effectiveness in humans. We found that a 20 mg kg^{-1} dose given daily starting on the day of arthritis induction significantly reduced the incidence of disease in DBA/1LacJ mice, and caused a small though statistically non-significant diminution of clinical signs in those animals that developed arthritis. Although the mice in this second study were given a booster immunization of type II collagen in IFA on day 28, the progression of the disease was slower and of less magnitude (i.e., no maximum severity scores >4) than was seen in the first collagen-induced arthritis study in which mice were only given the initial type II collagen immunization in CFA. It is possible that the type II collagen immunogen was partially denatured during the preparation process, or that the batch of CFA used was less potent. Nevertheless, the mice did present with clinical signs of arthritis that allowed us to easily score the progression of disease and to make valid comparisons between treatment groups.

Though an anti-arthritic effect of CM was demonstrated in the present study, the mode of action of this fatty acid alcohol ester remains to be discovered. While intact CM may be responsible for the observed anti-arthritic effects, it is possible that the ester is hydrolyzed in vivo and the 14-carbon free fatty acid (myristoleic acid) is the active pharmacologic agent. Although there are no reports of an anti-arthritic

effect of myristoleic acid, an anti-inflammatory effect has been demonstrated for oleic acid, a related *n*-9 fatty acid [12]. In addition, the 18:2*n*-6 fatty acid linoleic acid has been shown to have anti-arthritic effects in humans in some studies [13,14], although this has not been confirmed in others [15]. There is even more convincing evidence that dietary supplementation with *n*-3 and *n*-6 poly-unsaturated fatty acids from fish oils reduces inflammation and clinical signs in humans with rheumatoid arthritis [16–18]. The anti-inflammatory effect of fatty acids has been attributed to stabilization of cell membranes, inhibition of the formation of inflammatory mediators, and protection against oxidation [19,20]. Clearly, with the demonstration that CM has an anti-arthritic effect, further studies on its mode of action, pharmacokinetics, and pharmacodynamics are warranted.

Acknowledgements

This work was supported in part by an Applied Research Initiative Matching Grant between the State of Nevada and The Henry Rushing Fund. We thank Brandon Carter for able technical assistance.

References

[1] Diehl HW, May EL. Cetyl myristoleate isolated from Swiss albino mice: an apparent protective agent against adjuvant arthritis in rats. J Pharm Sci 1994;83:296–9.
[2] Edwards AM. CMO (cerasomol-*cis*-9-cetyl myristoleate) in the treatment of fibromyalgia: an open pilot study. J Nutr Environ Med 2001;11:105–11.
[3] Calder PC, Yaqoob P, Thies F, Wallace FA, Miles EA. Fatty acids and lymphocyte functions. Br J Nutr 2002;87:S31–48.
[4] Siegal S, Castellan Jr NJ. Nonparametric statistics for the behavioral sciences. 2nd ed. NewYork: McGraw-Hill; 1998, p. 38.
[5] Wooley PH, Luthra HS, Stuart JM, David CS. Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. J Exp Med 1981;154:688–700.
[6] Wooley PH. Collagen-induced arthritis in the mouse. Methods Enzymol 1988;162:361–73.
[7] Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen: an experimental model of arthritis. J Exp Med 1977;146:857–68.
[8] Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature 1980;283:666–8.
[9] DeLustro F, Carlson RP, Datko LJ, DeLustro B, Lewis AJ. The absence of antibodies to type II collagen in established adjuvant arthritis in rats. Agents Actions 1984;14:673–9.
[10] Kaibara N, Hotokebuchi T, Takagishi K, Katsuki I, Morinaga M, Arita C, et al. Pathogenetic difference between collagen arthritis and adjuvant arthritis. J Exp Med 1984;159:1388–96.
[11] Carlson RP, Datko LJ, O'Neill-Davis L, Blazek EM, DeLustro F, Beideman R, et al. Comparison of inflammatory changes in established type II collagen- and adjuvant-induced arthritis using outbred Wistar rats. Int J Immunopharmacol 1985;7:811–26.
[12] Martínez-Domínguez E, de la Puerta R, Ruiz-Gutiérrez V. Protective effects upon experimental inflammation models of a polyphenol-supplemented virgin olive oil diet. Inflamm Res 2001;50:102–6.
[13] Ariza-Ariza R, Mestanza-Peralta M, Cardiel MH. Omega-3 fatty acids in rheumatoid arthritis: an overview. Semin Arthritis Rheum 1998;27:366–70.
[14] Kremer JM. *N*-3 fatty acid supplements in rheumatoid arthritis. Am J Clin Nutr 2000;71:349S–51S.
[15] Nordstrom DC, Honkanen VE, Nasu Y, Antila E, Friman C, Konttinen YT. Alpha-linoleic acid in the treatment of rheumatoid arthritis. A double-blind, placebo-controlled and randomized study: flaxseed vs. safflower seed. Rheumatol Int 1995;14:231–4.
[16] Kremer JM, Lawrence DA, Petrillo GF, Litts LL, Mullaly PM, Rynes RI, et al. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal anti-inflammatory drugs; clinical and immune correlates. Arthritis Rheum 1995;38:1107–14.
[17] Lau CS, Morley KD, Belch JJF. Effects of fish oil supplementation on non-steroidal anti-inflammatory drug requirement in patients with mild rheumatoid arthritis—a double-blind placebo controlled study. Br J Rheumatol 1993;32:982–9.
[18] James MJ, Cleland LG. Dietary *n*-3 fatty acids and therapy for rheumatoid arthritis. Semin Arthritis Rheum 1997;27:85–97.
[19] Grimble RF. Modification of inflammatory aspects of immune function by nutrients. Nutr Res 1998;18:1297–317.
[20] Grimm H, Mayer K, Mayser P, Eigenbrodt E. Regulatory potential of *n*-3 fatty acids in immunological and inflammatory processes. Br J Nutr 2002;87:S57–9.