Clinical Nutrition (2007) **26**, 631–639

Available at www.sciencedirect.com

ScienceDirect

http://intl.elsevierhealth.com/journals/clnu

ORIGINAL ARTICLE

Examination of host immune resistance against *Listeria monocytogenes* infection in cyclophosphamide-treated mice after dietary lipid administration

Lidia Cruz-Chamorro, María A. Puertollano, Elena Puertollano, Gerardo Álvarez de Cienfuegos, Manuel A. de Pablo*

Unit of Microbiology, Department of Health Sciences, Faculty of Experimental Sciences, University of Jaén, E-23071 Jaén, Spain

Received 3 November 2006; accepted 27 June 2007

KEYWORDS
Fish oil;
Olive oil;
Hydrogenated coconut oil;
Cyclophosphamide;
Listeria monocytogenes;
Pro-inflammatory cytokines

Summary
Background & aims: Despite the beneficial effects in the resolution of inflammatory disorders due to their immunosuppressive properties, n-3 polyunsaturated fatty acids are associated with a reduction of immune resistance to some microorganisms. Here, we examine the influence of different dietary lipids on host immune resistance against *Listeria monocytogenes* in mice treated with cyclophosphamide (CPA).
Methods: Balb/c mice were fed one of four diets, which contained either olive oil (OO), fish oil (FO), hydrogenated coconut oil (HCO) or low fat (LF) for 4 weeks. Subsequently, mice were treated with CPA or PBS, prior to *L. monocytogenes* infection. Splenocyte proliferation, survival analysis, counts of viable bacteria from spleens and livers, and measurement of pro-inflammatory cytokine levels were determined.
Results: The FO-rich diet reduced survival, particularly in CPA-treated mice. CPA was responsible for a significant increase of viable bacteria recovery from spleens and livers within each group fed high fat diets, which was aggravated in mice fed an FO diet. In addition, a significant increase of both TNF-α and IL-12p70 levels was detected in this

Abbreviations: CFU, colony forming units; Con A, concanavalin A; CPA, cyclophosphamide; FCS, fetal calf serum; FO, fish oil; HCO, hydrogenated coconut oil; IL, interleukin; IFN, interferon; LF, low fat; LPS, lipopolysaccharide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; OD, optical density; OO, olive oil; PBS, phosphate buffered saline; TNF, tumor necrosis factor; TSA, tryptic soy agar

*Corresponding author. Facultad de Ciencias Experimentales, Departamento de Ciencias de la Salud, Universidad de Jaén, Área de Microbiología, E-23071-Jaén, Spain. Tel.: +34 953 212 003; fax: +34 953 212 943.
E-mail address: mapablo@ujaen.es (M.A. de Pablo).

0261-5614/$ - see front matter
doi:10.1016/j.clnu.2007.06.012

group. These results may acquire a crucial relevance in clinical nutrition, particularly when FO diets are administered to immunocompromised patients.
Conclusions: The mechanism(s) that impair(s) the elimination of *L. monocytogenes* could be associated with a low mitogen-stimulated splenocyte proliferation, and with an alteration of pro-inflammatory cytokine production. The application of the neutropenic agent CPA moderately aggravates the immunosuppressive state mainly in FO-fed animals.

Introduction

The extensive investigation concerned with the crucial role that several types of dietary fatty acids play in the modulation of immune system functions has clearly established that the administration of diets containing fish oil (FO) may produce important immunosuppressive effects in both animals and humans.[1,2] The n-3 polyunsaturated fatty acids (mainly contained in FO) have largely been defined as the most efficient immunomodulatory fatty acids, which have been involved in a suppression of lymphocyte proliferation,[3] a reduction of cytokine production,[4] an inhibition of natural killer cell activity,[5] or a diminution of serum antibody production such as IgG and IgM.[6] Therefore, the beneficial aspects of these types of fatty acids may be applied in the resolution of different autoimmune diseases characterized by an overactivation of the immune system.[7] Nevertheless, the prolonged utilization and the high administration of n-3 polyunsaturated fatty acids may confer a severe reduction of host immune resistance to infectious microorganisms as a result of their immunosuppressive properties. A large amount of information underlines the role of these fatty acids in the diminution of host defense against numerous bacteria, such as *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, viruses such as influenza virus, fungi or parasites.[8,9] Feeding mice with a diet containing long-chain n-3 polyunsaturated fatty acids reduces survival and increases bacterial recovery from both spleens and livers after an infectious challenge with *L. monocytogenes*, suggesting that this diet produces an immunosuppressive state responsible for the impairment of host immune resistance that hinders the efficient elimination of the microorganisms.[10,11] Recent studies have confirmed that one of the main reasons that explain the increase of host susceptibility to *L. monocytogenes* infection is the significant reduction of interleukin-(IL)-12 as well as interferon-γ (IFN-γ) production and IFN-γ signaling.[12–15] The immunosuppressive effects generated by diets containing FO may acquire a critical importance in patients at risk of infectious complications associated with their underlying disease or with an alteration of their immunological and nutritional status.

Despite the importance of a diet containing FO in the reduction of inflammatory disorders or in the diminution of cancer risk, little is known about the action of this and other fats in a model of mice treated with cyclophosphamide (CPA), and subsequently challenged with *L. monocytogenes*, a facultative intracellular pathogen that can originate a life-threatening infection in immunocompromised patients. To evaluate the action of different dietary lipids in severely immunosuppressed mice, we have used a model of chemotherapy constituted by CPA injections, which causes a delay in the onset of acquired cellular resistance, followed by an enhanced and slightly prolonged response in *L. monocytogenes* infected mice.[16] This study underscores the importance of several types of fatty acids to the potential application in clinical nutrition, particularly when lipid emulsions are administered to patients who are at risk of sepsis.

Materials and methods

Animals and diets

BALB/c mice 8–10 weeks old were purchased from the University of Jaen (breeding colony of Servicios Técnicos de Investigación, University of Jaén). They were housed in cages in an environmentally controlled room at a temperature of 24 °C with a 12-h light/12-h dark cycle. Mice were randomly allocated to receive one of four diets for 4 weeks and each group was allowed access *ad libitum* to water as well as to its respective diets. Experimental diets contained either olive oil (OO, 20%), fish oil (FO, 20%) or hydrogenated coconut oil (HCO, 20%). In addition, another group was fed a diet containing low fat (LF, 2.5% of fat), which was used as control. The diet containing HCO was supplemented with 1% of corn oil in order to prevent essential fatty acid deficiency. The composition of experimental diets is shown in Table 1.

Table 1 Composition (g/kg) of high fat and low fat experimental diets.*

Components	High fat	Low fat
Casein	200	200
D,L-Methionine	3	3
Corn starch	315	410
Sucrose	155	235
Fibre	80	80
Fats	200†	25
Mineral mix	35	35
Vitamin mix	10	10
Choline	2	2

*BALB/c mice were fed their respective diets for 4 weeks.
†High fat diets contained either olive oil (OO, 20%), fish oil (FO, 20%) or hydrogenated coconut oil (HCO, 20%).

Immunosuppression of mice with cyclophosphamide

The use of CPA (Sigma, St. Louis, MO, USA) represents a suitable alternative to the induction of immunosuppression, particularly with models of a short disease course.[17] At the end of the feeding period, each dietary group was divided into two subgroups. First one was injected with a dose of phosphate buffered saline (PBS), corresponding to non-treated mice, and the second one was injected with CPA (neutropenic mice) in order to generate a state of immunosuppression. This situation was induced in the animals by three subcutaneous injections of CPA at a concentration of 100 mg/kg body weight every 72 h. Finally, *L. monocytogenes* was injected 24 h after the last dose of CPA. The animal procedures complied with the national and European Union legislation on the care and use of animals and related codes of practice (86/609/EEC).

Preparation of *L. monocytogenes* and experimental infection

A virulent serum-resistant strain of *L. monocytogenes* was incubated on a blood agar plate at a temperature of 37 °C for 24 h. The bacteria were harvested by centrifugation and washed twice in saline solution. Finally, the concentration of bacteria was estimated optically at 550 nm and the number of viable bacteria was determined by counting developed colonies.

Mitogen-stimulated splenocyte proliferation

The proliferation of splenocytes was measured by the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) colorimetric assay. Briefly, spleens were removed aseptically and homogenized in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Austria) in the presence of HEPES, antibiotics and L-glutamine (Sigma). Cells were cultured in 96-well microtiter plates in triplicate, adjusted to a concentration of 10^5 per well, and incubated in a humidified atmosphere at 37 °C for 24 h in the presence of concanavalin A (Con A; Sigma) at a final concentration of 5 μg/ml or lipopolysaccharide (LPS; Sigma) from *Escherichia coli* serotype O26:B6 at a final concentration of 50 μg/ml. Splenocyte stimulation index was calculated as the optical density (OD) in the presence of mitogen (stimulated cells) divided by the OD in the absence of mitogen (unstimulated cells) as described previously.[18]

Measurement of survival

L. monocytogenes was suspended in PBS at a concentration of 10^6 CFU/ml. Then, 100 μl of this bacterial suspension was injected into each mouse through the tail vein. The survival was monitored every 8 h for 7 days after the *L. monocytogenes* challenge. Results were expressed as percentage of survival.

Determination of numbers of viable *L. monocytogenes* cells in the organs

After the injection of CPA, mice were challenged with 100 μl of a *L. monocytogenes* cell suspension (10^5 CFU/ml) through the tail vein. To evaluate the extent of systemic listerial infection, mice were sacrificed by cervical dislocation at 24 and 48 h post-infection, and the spleens and livers of infected animals were aseptically isolated in sterile PBS and weighed under sterile conditions. Then, spleen and liver cells were prepared by homogenizing these organs between frosted-glass slides in sterile distilled water. Thus, cells were disrupted by treatment with distilled water in order to lyse host cells and release intracellular bacteria. Then, serial 10-fold dilutions of each sample were made and an aliquot of 10 μl of each dilution was transferred onto blood tryptic soy agar (TSA, Scharlau Chemie, Barcelona, Spain) medium to determine the number of live *L. monocytogenes* in the spleens and livers. Plates were incubated at 37 °C for 24 h. Finally, the number of CFU was counted and the values were expressed as $\log_{10}$ viable bacteria.

Quantification of cytokine production by enzyme-linked immunosorbent assays (ELISA)

ELISA kits (R&D Systems, Minneapolis, MN, USA) were used for determination of IL-1β, IL-6, IL-12p70 and TNF-α concentration in the sera samples. Mice were fed their respective diets and divided into two subgroups. After the injection with CPA or PBS, mice were challenged with

Table 2 Body, spleen and liver weights of mice fed dietary lipids after 48 h of challenge with *L. monocytogenes*.

Diets	Body weight (g)		Spleen weight (g)				Liver weight (g)			
	−CPA	+CPA	−CPA	%*	+CPA	%*	−CPA	%†	+CPA	%†
LF	26.2±4.1	24.6±4.7	0.20±0.05	0.7±0.2	0.19±0.01^a	0.7±0.5^a	1.83±0.7^a	7.0±2.0	1.63±0.2^b	6.6±0.9^b
OO	24.1±1.3	22.6±5.2	0.22±0.01	0.9±0.07	0.12±0.04^b	0.5±0.2^{ab}	1.33±0.08^b	5.9±0.3	1.58±0.2^b	7.0±1.0^{ab}
FO	24.6±0.4	24.2±0.5	0.21±0.04	0.8±0.2	0.20±0.05^a	0.8±0.3^a	1.84±0.3^a	7.5±0.9	2.03±0.4^a	8.4±1.0^a
HCO	23.3±2.5	23.3±2.1	0.21±0.06	0.9±0.2	0.075±0.01^c	0.3±0.02^b	2.10±0.2^a	9.0±1.0	1.45±0.2^b	6.2±0.7^b

Data are mean±standard errors of the means of three independent experiments ($n = 5$ in each dietary group) and were analyzed by two-way ANOVA. Values in a column not sharing a common superscript letter are considered to be significantly different at $P<0.05$. LF, low fat; OO, olive oil, FO, fish oil; HCO, hydrogenated coconut oil. −CPA, non-treated mice; +CPA, cyclophosphamide-treated mice.

*Spleen weight as percentage of body weight.

†Liver weight as percentage of body weight.

L. monocytogenes (10^4 CFU per mouse). Mice were anesthetized with diethyl ether, and the blood was drawn from the retro-orbital plexus into tubes containing heparin (20 U/ml blood) at 24 and 48 h after infection. Serum was obtained after centrifugation of the tubes at 1500*g* for 30 min. Finally, sera samples were stored at −80 °C for subsequent analysis. Results were calculated against standard curves generated using known amounts of recombinant cytokines, according to the manufacturer's instructions in a microplate reader (BioRad) at a wavelength of 450 nm. Limits of detection for these assays were <3 pg/ml (IL-1β), 1.6 pg/ml (IL-6), <2.5 pg/ml (IL-12p70), and <5.1 pg/ml (TNF-α). Samples were assayed in duplicate.

Statistical analysis

Results are expressed as mean ± standard errors of the means. The effects of diets and CPA treatment and their interaction were analyzed by two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, the treatment means were compared using Fisher's least significant difference (LSD) test. Survival curves of *L. monocytogenes*-infected mice were compared using the Kaplan–Meier log rank test. The survival data from three independent experiments were pooled for statistical analysis. Viable bacteria counts from spleens or livers were $\log_{10}$ transformed before analysis. Differences were considered to be statistically significant at a value of $P<0.05$.

Results

Weight of body, spleens and livers from mice fed with experimental diets

After 48 h of the exposure to *L. monocytogenes* (10^5 CFU/ml), body weights between diet treatment groups were not significantly modified. Similarly, CPA treatment did not affect body weights (Table 2). The spleen weights of mice fed diets containing OO or HCO were considerably reduced in the CPA-treated group ($P<0.05$) (Table 2). Again, significant differences were observed among OO or HCO groups in comparison with values from the other groups in the relationship between CPA-treated and PBS-treated mice ($P<0.05$). Finally, the liver weight was significantly reduced in the group fed a diet containing OO and treated with PBS, whereas this value was notably increased in the group fed a diet containing FO and treated with CPA ($P<0.05$) (Table 2). Liver weight from mice fed an OO diet and treated with PBS was significantly reduced with respect to CPA-treated mice fed an FO diet ($P<0.05$).

Analysis of splenocyte proliferation

The treatment with PBS produced a significant increase of splenocyte proliferation in the group fed an OO diet and stimulated with Con A. In addition, a diminution of lymphocyte proliferation was observed in the group fed an OO diet, stimulated with LPS, and treated with CPA compared to values of PBS-treated mice ($P<0.05$). Figure 1 shows that splenocyte proliferation from mice fed a diet

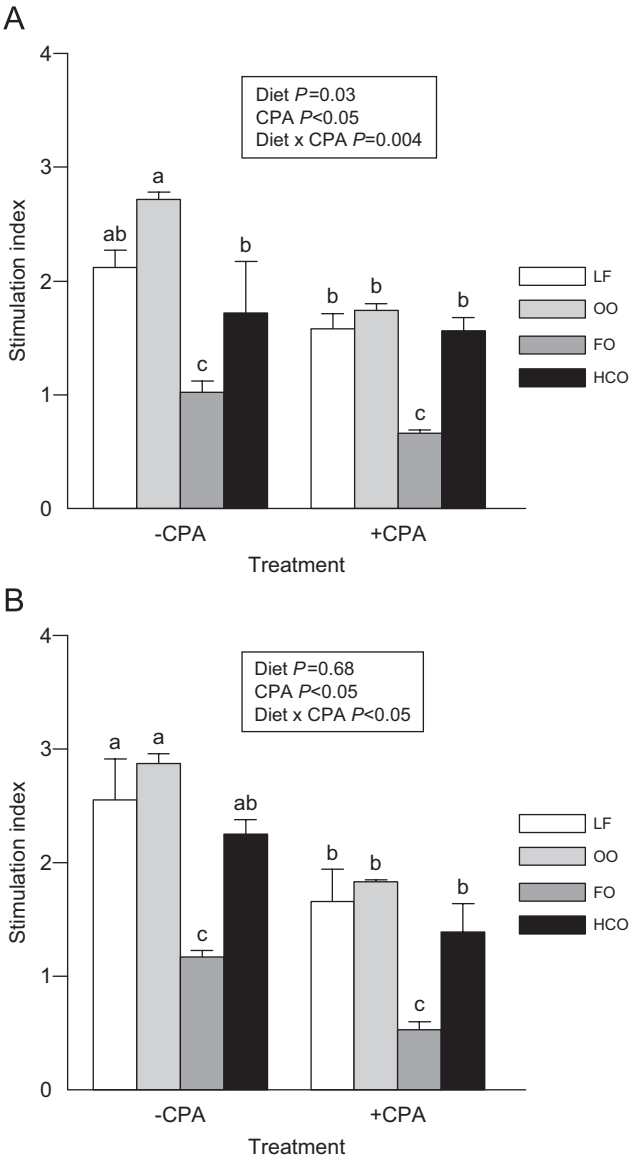


Figure 1 Analysis by the MTT colorimetric assay of the proliferative response of splenocytes to concanavalin A (Con A) and lipopolysaccharide (LPS) in mice fed dietary lipids and treated with cyclophosphamide (CPA). The splenocytes from non-treated mice and CPA-treated mice were distributed in culture medium into 96-flat-bottom plates and incubated for 24 h in the presence of Con A at a concentration of 5 μg/ml (A) or LPS from *E. coli* serotype O26:B6 at a concentration of 50 μg/ml (B). Subsequently, MTT was added to each well, and the cells were incubated for 4 h, as described in "Materials and methods" section. Results were expressed as the stimulation index ($n = 5$ mice in each group). Values are the mean ± ⊖ standard errors of the means of data in triplicate calculated as the optical density (OD) of cells treated in the presence of mitogen divided by the OD of cells incubated in the absence of mitogen. Data were analyzed by two-way ANOVA and insertions show the main effects and interactions. Different superscript letters are considered to be significantly different at $P<0.05$. LF, low fat; OO, olive oil; FO, fish oil; HCO, hydrogenated coconut oil; −CPA, non-treated mice; +CPA, cyclophosphamide-treated mice.

containing FO and stimulated in the presence of Con A (Figure 1A) or LPS (Figure 1B) was significantly reduced in comparison to values from the other groups ($P<0.05$), indicating that an FO diet was the most immunosuppressive. In general, the interactions (diet × treatment) have demonstrated significant differences between CPA-treated and PBS-treated groups when cells were stimulated in the presence of Con A ($P = 0.004$) or LPS ($P<0.05$).

Survival analysis

A lethal dose of a virulent *L. monocytogenes* strain was injected into each mouse to measure the resistance of animals in the defense against this infectious pathogen. The survival curves of mice fed different types of diets and experimentally infected with *L. monocytogenes* are illustrated in Figure 2. The data represent three pooled experiments with 15 mice/group. One day after the last treatment with PBS or CPA, PBS- and CPA-treated groups were challenged intravenously with *L. monocytogenes* and mortality was assessed for 7 days. Overall, a survival reduction was observed in the groups treated with CPA in comparison with survival data from PBS-treated mice. The survival curve of mice fed an FO diet at 2 days of infection differed from those fed this diet and treated with CPA ($P<0.05$), but all of animals died at 4 days after challenge (Figure 2C). Nevertheless, the survival rate of mice fed a diet containing OO was approximately of 60% after challenge with *L. monocytogenes*, and the treatment with CPA did not affect the host resistance to infection in this group (Figure 2B). Finally, the survival percentage of mice fed a diet containing HCO was lesser than the survival percentage in LF or OO groups, despite this reduction, we did not find

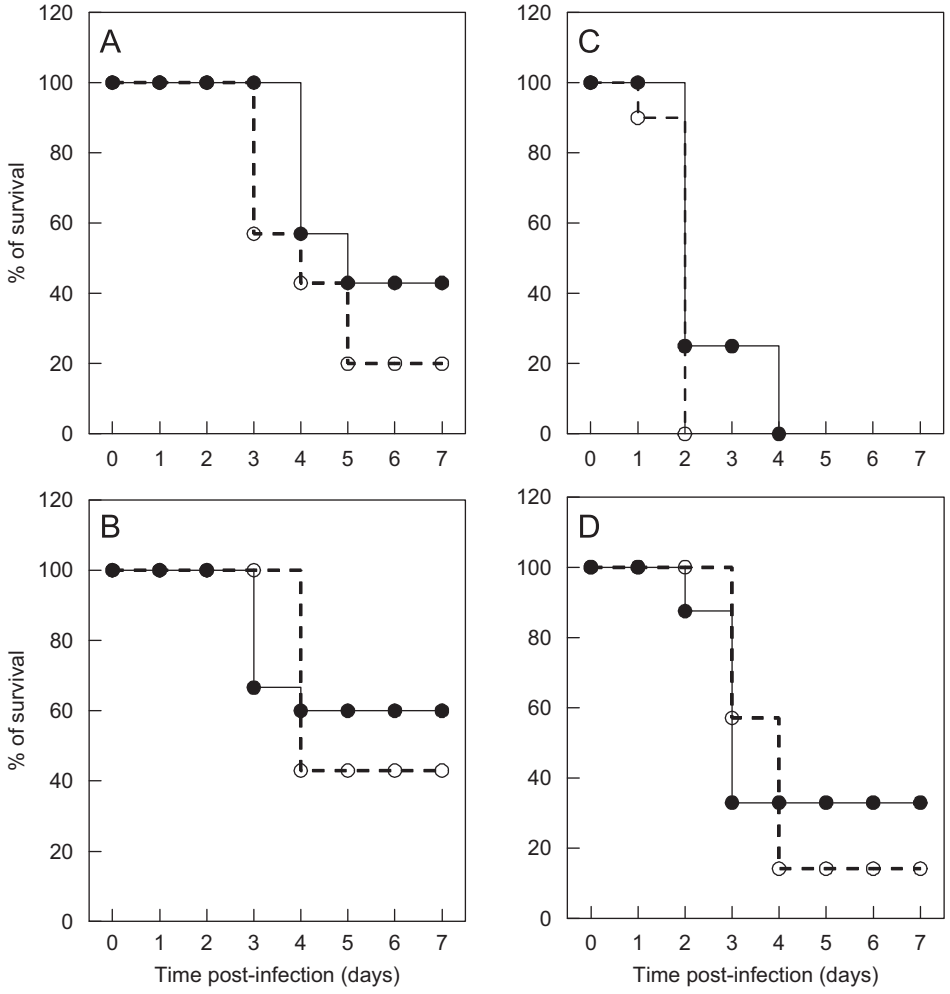


Figure 2 Measurement of survival percentage of mice fed dietary lipids after infection with *Listeria monocytogenes*. Mice were fed their respective diets for 4 weeks ($n = 15$ in each dietary group), treated with cyclophosphamide (CPA) and infected with *L. monocytogenes* (10^5 CFU per mouse). Closed circles represent the survival percentages of mice infected with *L. monocytogenes* (−CPA). Open circles represent the survival percentages of mice infected with *L. monocytogenes* and experimentally treated with CPA (neutropenic mice, +CPA). (A) Survival percentage of mice fed a low fat (LF) diet. (B) Survival percentage of mice fed an olive oil (OO) diet. (C) Survival percentage of mice fed a fish oil (FO) diet. (D) Survival percentage of mice fed a hydrogenated coconut oil (HCO) diet. The data represent the pooled result of three experiments.

Table 3 Recovery of viable bacteria from spleens and livers of mice fed dietary lipids after 24 h of challenge with *Listeria monocytogenes*.

Diets	Mean $\log_{10}$ CFU of *L. monocytogenes* ± S.E.M.			
	Spleen		Liver	
	−CPA	+CPA	−CPA	+CPA
LF	2.9 ± 0.3^{b}	3.0 ± 0.7^{c}	3.5 ± 1.4^{c}	3.8 ± 1.8^{b}
OO	3.0 ± 1.0^{b}	3.6 ± 1.6^{b}	3.9 ± 1.4^{b}	4.0 ± 1.2^{b}
FO	4.6 ± 1.7^{a}	6.1 ± 1.2^{a}	6.3 ± 1.4^{a}	7.4 ± 2.8^{a}
HCO	2.3 ± 0.9^{c}	3.1 ± 1.1^{c}	3.2 ± 1.3^{c}	3.7 ± 1.8^{b}

Data are mean ± standard errors of the means of three independent experiments after logarithmic ($\log_{10}$) transformation of these variables ($n = 5$ in each dietary group) and were analyzed by two-way ANOVA. Values in a column not sharing a common superscript letter are considered to be significantly different at $P < 0.05$. LF, low fat; OO, olive oil; FO, fish oil; HCO, hydrogenated coconut oil. −CPA, non-treated mice; +CPA, cyclophosphamide-treated mice.

Table 4 Recovery of viable bacteria from spleens and livers of mice fed dietary lipids after 48 h of challenge with *Listeria monocytogenes*.

Diets	Mean $\log_{10}$ CFU of *L. monocytogenes* ± S.E.M.			
	Spleen		Liver	
	−CPA	+CPA	−CPA	+CPA
LF	3.5 ± 1.6^{c}	3.8 ± 1.1^{c}	3.0 ± 0.9^{d}	3.2 ± 1.2^{d}
OO	4.3 ± 2.0^{b}	4.6 ± 1.8^{b}	4.5 ± 2.1^{c}	5.0 ± 1.8^{c}
FO	6.3 ± 2.5^{a}	6.7 ± 1.8^{a}	5.9 ± 1.1^{a}	7.0 ± 2.1^{a}
HCO	6.0 ± 2.1^{a}	6.5 ± 2.6^{a}	5.3 ± 2.0^{b}	6.2 ± 2.3^{b}

Data are mean ± standard errors of the means of three independent experiments after logarithmic ($\log_{10}$) transformation of these variables ($n = 5$ in each dietary group) and were analyzed by two-way ANOVA. Values in a column not sharing a common superscript letter are considered to be significantly different at $P < 0.05$. LF, low fat; OO, olive oil; FO, fish oil; HCO, hydrogenated coconut oil. −CPA, non-treated mice; +CPA, cyclophosphamide-treated mice.

statistically significant differences (Figure 2D), whereas a substantial reduction of survival was observed in LF group treated with CPA at 7 days of infection (Figure 2A) ($P < 0.05$).

Quantitation of bacterial load in spleens and livers of *Listeria*-challenged mice

Here, the number of viable bacteria recovered from the spleens and livers of CPA-treated mice fed a diet containing FO at 24 h (Table 3) and 48 h (Table 4) following the challenge with *L. monocytogenes* was substantially higher than in PBS-treated mice ($P < 0.05$). Compared to PBS-treated mice, CPA-treated mice at 24 h had approximately 30- and 10-fold higher counts of bacteria in their spleen and liver, respectively. However, these values were modestly reduced at 48 h of challenge in the spleen (2.5-fold), whereas the differences between the two treatment groups were maintained in the liver (10-fold).

Measurements of pro-inflammatory cytokine production

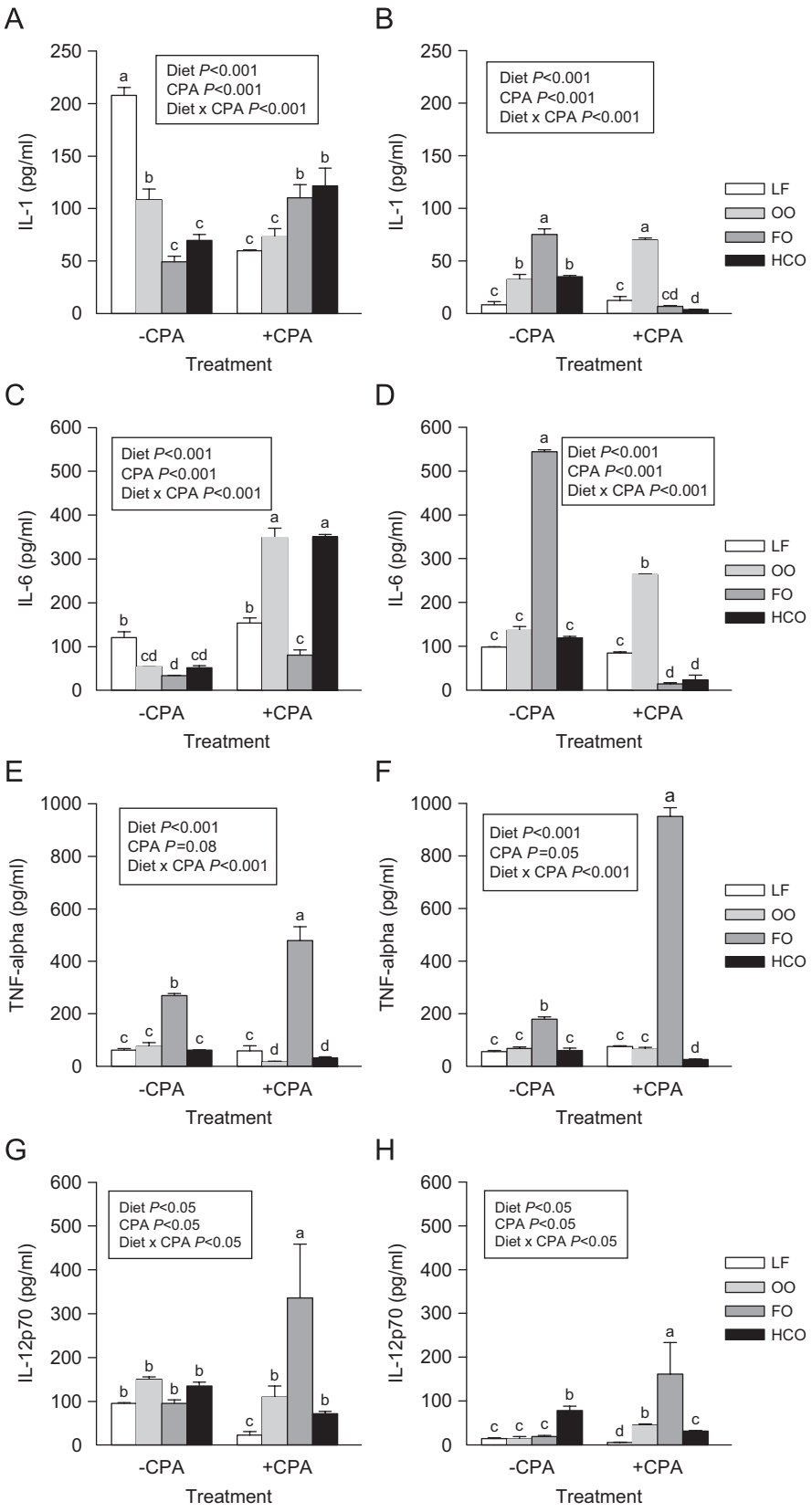
The effects of dietary lipids on pro-inflammatory cytokine concentration in the sera of mice treated and non-treated with CPA are shown in Figure 3. IL-1β levels from mice fed FO and HCO diets were significantly increased with respect to LF, and OO groups in CPA-treated mice at 24 h of *L. monocytogenes* infection ($P < 0.05$). By contrast, IL-1β concentration from mice fed FO and HCO diets and treated with CPA was dramatically reduced at 48 h of infection ($P < 0.001$). An increase of IL-6 concentration was observed in the serum from mice fed OO and HCO diets and treated with CPA at 24 h of infection ($P < 0.001$). It is important to remark the substantial increase of IL-6 concentration detected in the mice fed an FO diet and PBS-treated at 48 h of challenge in comparison with values of mice treated with CPA ($P < 0.001$). Surprisingly, the concentration of TNF-α from mice fed an FO diet and treated with CPA (+CPA) at 24 h, and particularly at 48 h of infection was significantly increased with respect to PBS-treated groups (−CPA) ($P < 0.001$). Similarly, IL-12p70 levels were significantly increased at 24 and 48 h of infection in mice fed an FO diet and treated with CPA ($P < 0.05$).

Discussion

This investigation confirms previous results supporting the notion that a diet containing FO is responsible for an immunosuppressive effect, leading to a diminution of host immune resistance.[8,9] Nevertheless, few studies have described the action of dietary lipids in immunosuppressed organisms. Therefore, this research constitutes a new advance that contributes to the clarification of the

Figure 3 Measurement of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-12p70 (IL-12p70) concentration from the sera of peripheral blood of mice fed with different dietary lipids. BALB/c mice ($n = 5$ in each dietary group) were fed either a low fat (LF) diet, an olive oil diet (OO), a fish oil diet (FO) or a hydrogenated coconut oil diet (HCO) and treated with cyclophosphamide (+CPA) or PBS (−CPA), prior to *L. monocytogenes* infection (10^4 CFU per mouse). The concentration of IL-1β (A, B), IL-6 (C, D), TNF-α (E, F) and IL-12p70 (G, H) were measured at 24 h (A, C, E, G) and 48 h (B, D, F, H) after bacterial infection. Quantification of pro-inflammatory cytokine levels in experimental samples was made by extrapolation from ELISA results, using various concentrations of rIL-1β, rIL-6, rTNF-α and rIL-12p70, respectively, as a standard. Results are means ± standard errors of the means of two independent determinations in duplicate. Data were analyzed by two-way ANOVA and insertions show the main effects and interactions. Different superscript letters are considered to be significantly different at $P < 0.05$.

established relationship among dietary lipids, immune system and infection. Here, we demonstrate that the administration of an FO-rich diet in immunocompromised animals, produces an exacerbated reduction of host natural resistance against the intracellular growth pathogen *L. monocytogenes*, in spite of an efficient response in the



production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α). To generate an immunosuppressive state after dietary lipid administration, mice were previously treated with CPA, prior to *L. monocytogenes* infection. CPA has been described as an immunosuppressive agent capable of diminishing the capacity of murine peritoneal macrophages to produce TNF and IL-1.[17] In this model of neutropenia caused by CPA injections, it was reported that the neutrophil counts are very low, as well as the number of lymphocytes and monocytes.[19] In fact, acute treatment with this immunosuppressive agent depresses resistance to the microbial infections with *L. monocytogenes* and other microbial pathogens.[20] Human studies have applied this immunosuppressive agent in the treatment of neoplastic diseases,[21] and in general in the treatment of autoimmune disorders[22] due to its immunosuppressive and chemotherapeutic properties. Nonetheless, our results have demonstrated that CPA treatment is involved in a general alteration of pro-inflammatory cytokine concentration after dietary lipid administration. In fact, both TNF-α and IL-12p70 levels were significantly increased in the serum from mice fed a diet containing FO, which had previously been treated with CPA. In a strict contrast, the concentration of both IL-1β and IL-6 from the CPA-treated group was substantially reduced at 48 h of challenge in mice fed an FO diet. Therefore, the reduction of mice survival and the increase of bacterial recovery from spleens and livers of mice fed an FO diet and treated with CPA in this model of immunosuppression cannot be explained by an inefficient pro-inflammatory cytokine production after the quantification of IL-1β, IL-6, IL-12p70 and TNF-α.

The participation of neutrophils in the host defense against intracellular pathogenic bacteria has been well established. These cells exert a critical function in order to prevent the dissemination of *Listeria* during the early stages of infection.[23] However, there is limited information about the interactions among dietary lipid administration, neutropenic depletion and resistance to this intracellular microorganism in immunosuppressed animals. A recent investigation has reported that the impairment of host resistance associated with n-3 polyunsaturated fatty acid administration is independent of neutrophil activity, when *L. monocytogenes*-infected mice were treated with the neutrophil-depleting drug RB6-8C5.[24] Our results reveal that CPA significantly alters mice survival and the bacterial loads from both spleens and livers in the four groups and particularly in mice fed a diet containing FO at 24 h of challenge (30- and 10-fold, respectively). Although CPA administration causes neutropenia,[19] early investigations demonstrated that CPA may also affect T and B cell functions, leading to an immunosuppressive state.[25–27] Irrespective of this action, we speculate that the pathways of bacterial inoculation may be responsible in part for the differential effects observed in our study. This experimental design used an intravenous injection as site of infection, whereas Fritsche et al.[24] have applied an intraperitoneal route in their investigation. We have also confirmed a reduction of splenocyte proliferation in the group fed an FO diet after the stimulation of splenocytes with either Con A and LPS,[18] but the lymphocyte proliferation in this group was not significantly modified after the treatment with CPA, although it is important to underline the moderate reduction of splenocyte proliferation observed after the exposure to this immunosuppressive agent in the four groups examined. In fact, a previous study indicated that the immunosuppression caused by CPA is related to polyclonal deletion of mitogen-stimulated T lymphocytes,[26] which was impaired during murine listeriosis.[28]

Less attention has been paid to the action of monounsaturated fatty acids on the immune system. It is clear that an OO-rich diet exerts a modulatory effect upon immune functions due mainly to the action of oleic acid (the main fatty acid contained in this fat),[29] however, these immunosuppressive events are not as marked as those caused by FO. Thus, we hypothesize that the administration of an OO diet to CPA-treated mice does not produce a reduction as severe in the host immune defense as an FO diet. As mentioned previously, FO diets reduce host immune resistance by a significant diminution of bactericidal capacity,[30] and by a modulation or no alteration of different types of cytokines such as IL-12, IL-4, TNF-α and IFN-γ.[12–15] These results indicate that survival percentage of mice to *L. monocytogenes* infection was diminished in the group fed an FO diet, but the reduction of this percentage was more drastic in CPA-treated mice, because all of animals died at 2 days of the exposure to *L. monocytogenes*, whereas survival percentage were higher in OO group than in FO group. Initially, these effects could be attributed to a significant reduction of T and B cell response in mice fed an FO diet, as corroborated by a diminution of mitogen-stimulated splenocyte proliferation when these data were compared with those from other groups.

Hence, it is necessary to find a balance that allows the resolution of inflammatory disorders without adversely affecting host immune defense. Irrespective of the importance of n-3 polyunsaturated fatty acids in the resolution of inflammatory disorders, our present results suggest that an FO diet administered in high doses is not only involved in a reduction of immune system functions, but also exacerbates the immunosuppressive state in CPA-treated mice (survival reduction and bacterial recovery increase), although this agent does not diminish the levels of pro-inflammatory cytokines in all of the groups fed dietary lipids. Indeed, the concentration of both TNF-α and IL-12p70 was substantially increased in the group fed an FO diet after CPA treatment, whereas a recent study suggests that this diet does not affect *in vivo* TNF-α production during the early phases of listeriosis.[24] Therefore, we consider that the reduction of host natural resistance cannot be exclusively attributed to an inadequate pro-inflammatory cytokine production.

Numerous studies have related to both FO and OO as dietary elements of enormous biological value in the human health, which may play an important role in clinical nutrition.[31,32] On the basis of these and other experimental observations, we estimate that future studies should determine the properties of these fats on immune functions and the impact of them on host immune defense, because they could constitute a potential alternative to other fats applied in parenteral lipid emulsions.

Acknowledgments

We thank the Ministry of Education and Science (Grant no. AGL2005-00605) and Fundación Citoliva (Centro de

Innovación y Tecnología del Olivar y del Aceite) for supporting this investigation. E.P. receives a predoctoral fellowship from the Ministry of Education and Science (Spain). L.C.C., M.A.P. and E.P. carried out the collection and samples analyses. M.A.P., G.A.dC., and M.A.dP. contributed in the design of the study and carried out the analysis of data. M.A.P and M.A.dP. conceived of the study, coordinated and drafted the manuscript. M.A.dP. approved the final version. All authors read and approved the final manuscript.

References

1. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids* 2000;**36**:1007–24.
2. de Pablo MA, Alvarez de Cienfuegos G. Modulatory effects of dietary lipids on immune system functions. *Immunol Cell Biol* 2000;**78**:31–9.
3. Yaqoob P, Newsholme EA, Calder PC. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunology* 1994;**82**:603–10.
4. Wallace FA, Miles EA, Evans C, Stock TE, Yaqoob P, Calder PC. Dietary fatty acids influence the production of Th1- but not Th2-type cytokines. *J Leukoc Biol* 2001;**69**:449–57.
5. Yaqoob P, Newsholme EA, Calder PC. Inhibition of natural killer cell activity by dietary lipids. *Immunol Lett* 1994;**41**:241–7.
6. Xi S, Cohen D, Chen LH. Effects of fish oil on cytokines and immune functions of mice with murine AIDS. *J Lipid Res* 1998; **39**:1677–87.
7. Kelley DS. Modulation of human immune and inflammatory responses by dietary fatty acids. *Nutrition* 2001;**17**:669–73.
8. Anderson M, Fritsche KL. (n-3) Fatty acids and infectious disease resistance. *J Nutr* 2002;**132**:3566–76.
9. de Pablo MA, Puertollano MA, Alvarez de Cienfuegos G. Biological and clinical significance of lipids as modulators of immune system functions. *Clin Diagn Lab Immunol* 2002;**9**: 945–50.
10. de Pablo MA, Puertollano MA, Galvez A, Ortega E, Gaforio JJ, Alvarez de Cienfuegos G. Determination of natural resistance of mice fed dietary lipids to experimental infection induced by *Listeria monocytogenes*. *FEMS Immunol Med Microbiol* 2000;**27**:127–33.
11. Fritsche KL, Shahbazian LM, Feng C, Berg JN. Dietary fish oil reduces survival and impairs bacterial clearance in C3H/Hen mice challenged with *Listeria monocytogenes*. *Clin Sci (Lond)* 1997;**92**:95–101.
12. Fritsche KL, Anderson M, Feng C. Consumption of eicosapentaenoic acid and docosahexaenoic acid impair murine interleukin-12 and interferon-gamma production in vivo. *J Infect Dis* 2000;**182**:S54–61.
13. Puertollano MA, Cruz-Chamorro L, Puertollano E, Perez-Toscano MT, Alvarez de Cienfuegos G, de Pablo MA. Assessment of interleukin-12, gamma interferon, and tumor necrosis factor alpha secretion in sera from mice fed with dietary lipids during different stages of *Listeria monocytogenes* infection. *Clin Diagn Lab Immunol* 2005;**12**:1098–103.
14. Puertollano MA, Puertollano E, Ruiz-Bravo A, Jimenez-Valera M, de Pablo MA, Alvarez de Cienfuegos G. Changes in the immune functions and susceptibility to *Listeria monocytogenes* infection in mice fed dietary lipids. *Immunol Cell Biol* 2004;**82**:370–6.
15. Irons R, Fritsche KL. Omega-3 polyunsaturated fatty acids impair in vivo interferon-gamma responsiveness via diminished receptor signaling. *J Infect Dis* 2005;**191**:481–6.
16. Kerckhaert JA, Hofhuis FM, Willers JM. Influence of cyclophosphamide on delayed hypersensitivity and acquired cellular resistance to *Listeria monocytogenes* in the mouse. *Immunology* 1977;**32**:1027–32.
17. Marcinkiewicz J, Bryniarski K, Ptak W. Cyclophosphamide uncovers two separate macrophage subpopulations with opposite immunogenic potential and different patterns of monokine production. *Cytokine* 1994;**6**:472–7.
18. Puertollano MA, de Pablo MA, Alvarez de Cienfuegos G. Relevance of dietary lipids as modulators of immune functions in cells infected with *Listeria monocytogenes*. *Clin Diagn Lab Immunol* 2002;**9**:352–7.
19. Van't Wout JW, Linde I, Leijh PCJ, van Furth R. Effect of irradiation, cyclophosphamide and etoposide (VP-16) on number of peripheral blood and peritoneal leukocytes in mice under normal conditions and during acute inflammatory reaction. *Inflammation* 1989;**13**:1–14.
20. Morahan PS, Bradley SG, Munson AE, Duke S, Fromtling RA, Marciano-Cabral F. Immunotoxic effects of diethylstilbestrol on host resistance: comparison with cyclophosphamide. *J Leukoc Biol* 1984;**35**:329–41.
21. Tisdale JF, Dunn DE, Maciejewski J. Cyclophosphamide and other new agents for the treatment of severe aplastic anemia. *Semin Hematol* 2000;**37**:102–9.
22. Martinez-Maza O, Moody DJ, Rezai AR, Ellison GW, Myers LW, Tourtellotte WW, et al. Increased spontaneous immunoglobulin secretion associated with cyclophosphamide-induced immune suppression. *J Clin Immunol* 1987;**7**:107–13.
23. Rogers HW, Unanue ER. Neutrophils are involved in acute, nonspecific resistance to *Listeria monocytogenes* in mice. *Infect Immun* 1993;**61**:5090–6.
24. Fritsche KL, Irons R, Pompos L, Janes J, Zheng Z, Brown C. Omega-3 polyunsaturated fatty acid impairment of early host resistance against *Listeria monocytogenes* infection is independent of neutrophil infiltration and function. *Cell Immunol* 2005; **235**:65–71.
25. Cupps TR, Edgar LC, Fauci AS. Suppression of human B lymphocyte function by cyclophosphamide. *J Immunol* 1982; **128**:2453–7.
26. Kondratyeva TK, Fontalin LN, Mikheeva NV. T-cell immunodeficiency induced by T-cell mitogens combined with cyclophosphamide injections. *Immunol Lett* 1992;**34**:71–7.
27. Zhu LP, Cupps TR, Whalen G, Faucdi AS. Selective effects of cyclophosphamide therapy on activation, proliferation, and differentiation of human B cells. *J Clin Invest* 1987;**79**: 1082–90.
28. Chan YY, Cheers C. Recovery from T cell depletion during murine listeriosis and effect on a T-dependent antibody response. *Infect Immun* 1982;**38**:694–8.
29. Jeffery NM, Yaqoob P, Newsholme EA, Calder PC. The effects of olive oil upon rat serum lipid levels and lymphocyte functions appear to be due to oleic acid. *Ann Nutr Metab* 1996;**40**: 71–80.
30. Puertollano MA, de Pablo MA, Alvarez de Cienfuegos G. Immunomodulatory effects of dietary lipids alter host natural resistance of mice to *Listeria monocytogenes* infection. *FEMS Immunol Med Microbiol* 2001;**32**:47–52.
31. Lanza-Jacoby S, Flynn JT, Miller S. Parenteral supplementation with a fish-oil emulsion prolongs survival and improves rat lymphocyte function during sepsis. *Nutrition* 2001;**17**: 112–6.
32. Moussa M, Le Boucher J, Garcia J, Tkaczuk J, Ragab J, Dutot G, et al. In vivo effects of olive oil-based lipid emulsion on lymphocyte activation in rats. *Clin Nutr* 2000;**19**:49–54.