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Long-Chain Polyunsaturated Fatty Acids Modulate Lung Inflammatory Response Induced by *Pseudomonas aeruginosa* in Mice

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ABSTRACT

Polyunsaturated fatty acid (PUFA) immunomodulatory properties have been studied extensively in chronic infections. Few studies have focused on acute infection; thus, PUFA effects in a mouse model of *Pseudomonas aeruginosa* (PA)-induced lung injury were evaluated. C57BL/6 mice were randomized to be fed for 3 wk with an eicosapentaenoic acid (EPA) diet, an arachidonic acid (AA) diet, or a control diet [saturated fatty acids]. Lung injury was induced by intratracheal instillation of 10^7 CFU of PA per mouse. In each diet group, animals were studied either without or after PA–inducing lung injury. Evaluation criteria were early mortality; inflammatory response assessed with tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and IL-10 levels in bronchoalveolar lavage; lung injury evaluation; and extravascular lung water, assessed 24 h after the injury. After PA-induced lung injury, no difference in early mortality was observed; TNF-α level was significantly higher in the EPA diet than in the other two diet groups. No difference for the other cytokines was found among the groups. Lung edema was also more important in the EPA group, consistent with the variations of TNF-α levels. Our study clearly shows that in PA-induced acute lung injury, n-3 PUFA induces differences in the inflammatory response with a higher level of lung edema. Modulation of the inflammatory response with n-3 PUFA can influence the response to a bacterial challenge. **(*Pediatr Res* 58: 211–215, 2005)**

Abbreviations

AA, arachidonic acid
AIN, American Institute of Nutrition
BAL, bronchoalveolar lavage
EPA, eicosapentaenoic acid
PA, Pseudomonas aeruginosa
PUFA, polyunsaturated fatty acid
Q_B, weight of intrapulmonary blood
Q_D, dry lung weight
Q_W, wet lung weight
SF, saturated fatty acids
TNF-α, tumor necrosis factor-α
W/D, wet to dry weight ratio

The effects of polyunsaturated fatty acids (PUFAs) in the modulation of the inflammatory response are now well established (1,2). Different authors showed after an omega 3 fatty acid diet that isolated peripheral blood mononuclear cells had a decreased *ex vivo* production of tumor necrosis factor-α (TNF-α), IL-1, and IL-6 (3–5). Several hypotheses have been raised to explain this phenomenon; the most interesting is based on the biochemical properties of eicosapentaenoic acid (EPA). EPA can be converted to the 3-series prostaglandins and 5-series leukotrienes (6); these mediators are considered less biologically active and less inflammatory than the arachidonic acid (AA)-derived 2-series prostaglandins and 4-series leukotrienes (7,8). Lung functional consequences of PUFA supplementation have also been studied omega 3 fatty acid can reduce both lung membrane injury (9) and lung edema accumulation (10–12). As a consequence, an improvement of the survival rate has also been shown after infection caused by different microorganisms (13–15). These models used different types of bacteria, including Gram-negative bacilli; among them, *Pseudomonas aeruginosa* (PA) has never been studied.

PA is frequently encountered in intensive care units, being responsible for nosocomial pneumonia (16). This pathogen is associated with a high mortality rate, reaching 70% in some studies (17). Alteration in dietary lipid composition in the

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intensive care unit patients could be an attractive therapeutic target to modulate the host response.

The aim of this study was to determine the effect of PUFAs in a murine model of acute lung injury induced by PA. To evaluate the benefit of a preventive diet, we analyzed the inflammatory response at baseline and after the injury, as well as the alveolar capillary barrier permeability and lung edema.

METHODS

Animals. Male C57BL/6 mice (6 wk of age), purchased from Charles-River, were housed in the Lille University animal care facility and fed with three different diets produced by Numico Research (Friedrichsdorf, Germany). Food and water were available *ad libitum*. All mice were maintained in a protected unit. All experiments were performed with approval of the Lille institutional animal care and use committee.

Foods. The basis of our diets was adapted from the AIN-93 (American Institute of Nutrition) rodent diet (18). We used the rodent diet formulated for growth (AIN-93G; Table 1). We substituted AIN-93G lipids (soybean oil) by three different isocaloric fatty acid mixtures: EPA, AA, and saturated fatty acids (SF; Table 2).

Design of the study. Mice first were randomized in one of the three diet groups for 3 wk (Fig. 1). All experiments were performed blindly. A second randomization decided whether the animal would be infected with PA. Data analysis was performed 24 h after the instillation. Twenty mice were used in each group (10 for the inflammatory response and 10 for lung physiologic studies).

Experimental model of acute lung injury. PA (PAO1 strain) was incubated in 125 mL of tryptic soy broth at 37°C in a rotating shaking water bath for 8 h. The culture then was washed twice with PBS and resuspended in PBS. The resulting bacterial suspension at 1×10^9 CFU/mL was diluted at 1:10 (10^8 CFU/mL). Mice were anesthetized with sevoflurane (Servorane; Abbott, UK) and placed in dorsal recumbency. Acute lung injury was produced according to the method described by Pennington and Ehrie (19). Transtracheal insertion of a 24-G animal feeding needle was used to instill 100 μL of the inoculum (10^7 CFU/mouse).

Evaluation of lung inflammatory response. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea *in situ* with a 22-G, 1.5-inch bead-tipped feeding needle; four aliquots of 0.5 mL of sterile PBS were instilled; and the fluid was collected by aspiration. BAL samples were filtered and immediately frozen at −80°C after collection. Murine TNF-α, IL-1, IL-6, and IL-10 were measured using commercially available sandwich enzyme immunoassays (Quantikine; R&D Systems, Abingdon OX, UK). BAL samples were assayed in duplicate and compared with known standards.

Evaluation of endothelial injury: measurement of the alveolar capillary membrane permeability. To evaluate the endothelial injury, we calculated the albumin flux across the endothelial barrier using a previously described method adapted from rats (20). Briefly, 2 h before the experimentation, 0.5 mL of ^{125}I-labeled human serum albumin (HAS; 1 μCi; CIS Biointernational, Gif sur Yvette, France) was injected intraperitoneally. After i.p. injection of pentobarbital sodium (Sanofi, Libourne, France), exsanguination was performed through the jugular vein. The lungs were removed through a sternotomy, and blood radioactivity and Hb were measured.

Lung weight and radioactivity count were measured before homogenization (polytron, PT 1600 E; Fisher Bioblock Scientific, Switzerland) and centrifugation. The supernatant Hb content was measured. Blood and lung homogenate samples were placed at 37°C during 7 d to determine the wet to dry weight ratio (W/D).

Table 1. *Diet composition in gram per kilogram of diet*

Ingredient	AIN-93G (g/kg diet)
Cornstarch	397
Casein	200
Dextrinized cornstarch	132
Sucrose	100
Soybean oil	70
Fiber	50
Mineral mix	35
Vitamin mix	10
L-cystine	3
Choline bitartrate	2.5

Table 2. *Repartition of each fatty acid in the three diet groups (gram per 100 grams of lipids)*

Fatty acid	EPA diet	AA diet	SF Diet
C16:0 (PA)	9.0	20.0	30.0
C18:0 (SA)	3.0	7.0	10.0
Total of saturated FA	12.0	27.0	40.0
MUFA oleic acid	45.0	45.0	33.0
C18:2 ω6 (LA)	23.0	23.0	27.0
C18:3 ω3 (ALA)	10.0	0.1	0.0
C18:3 ω6 (GLA)	3.0	0.1	0.0
C18:4 ω3 (STA)	2.0	0.1	0.0
C20:4 ω6 (AA)	0.5	5.0	0.0
C20:5 ω3 (EPA)	5.0	0.5	0.0
C22:6 ω3 (DHA)	2.0	0.2	0.0
Total of PUFA	43.0	28.0	27.0

MUFA, mono-unsaturated fatty acid; PA, palmitic acid; SA, stearic acid; LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; STA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; SF, Control diet with saturated fatty acids.

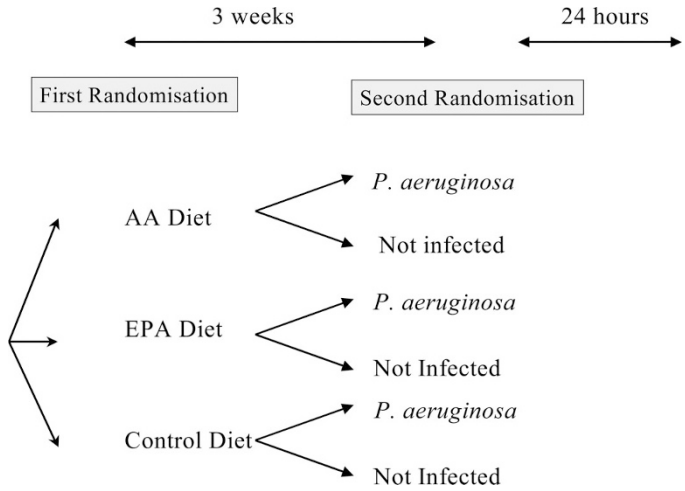

Figure 1. Design of the study.

Permeability. We defined

Extravascular lung water. Lung W/D was determined by removing the lung at the end of the experiment and recording the wet weight. The lung then was placed in a 37°C incubator for 7 d, at which time the dry weight was recorded. For each pair of lungs, the W/D then was calculated.

Lung histopathology. Lungs were fixed in 2% paraformaldehyde for at least 48 h, then dissected midsagittally and embedded in paraffin. Serial lung sections (5 μm thick) were stained with hematoxylin-eosin using standard techniques and examined for histopathologic changes. Diffuse alveolar damage was evaluated using a blind histologic scoring technique; three animals were analyzed in each group. Lung samples were divided into 20 observation fields at ×40 magnification and graded on a scale of 0 to 3 (0 = absent, 1 = mild, 2 = moderate, and 3 = intense) for inflammation. The lung injury was described as focal or diffuse for each sample.

Statistical Analysis. Data are expressed as the mean± SEM. Kruskall-Wallis one-way ANOVA using Dunn's method to compare differences between groups and Mann-Whitney rank sum test analysis were performed using SPSS (11.0.1 version; LEAD Technologies, Chicago, IL). Statistical significance was considered at $p \leq 0.05$.

RESULTS

Basal inflammatory response was comparable between groups. After 3 wk of diet before PA acute infection, all groups were comparable for all of the studied variables. There was no significant difference among the three diet groups regarding cytokine levels, lung permeability, and W/D (Table 3).

Table 3. *Secretion of cytokines and physiological parameters in the different diet groups without* Pseudomonas aeruginosa *infection*

	EPA Diet (n = 10)	AA Diet (n = 10)	SF Diet (n = 10)	p
IL1β (pg/ml) in BAL	0	0	0	—
IL6 (pg/ml) in BAL	0	0	0	—
IL10 (pg/ml) in BAL	0	0	0	—
TNFα (pg/ml) in BAL	0	0	0	—
Indexed Permability	0.73 ± 0.23	0.84 ± 0.28	0.84 ± 0.46	0.77
W/D	4.71 ± 0.36	4.64 ± 1.06	4.74 ± 0.51	0.95

IL, interleukin; BAL, Broncho-alveolar liquid; I Perm, indexed permeability; W/D, wet to dry weight lung ratio. Results are expressed as means and standard error of the mean.

PA instillation significantly increased cytokine levels in the BAL. Independent of the diet group, there was a significant difference for all cytokine measurements between controls and PA-infected animals. Alveolar capillary membrane permeability also increased in infected groups (Fig. 2). Mortality rates were 20, 27, and 13% for the EPA, AA, and SF diet groups, respectively (NS).

EPA increased TNF production and W/D after PA infection. TNF-α levels in the BAL were, respectively, 1752, 1171, and 578 pg/mL for the EPA, AA, and SF diets ($p = 0.01$; Fig. 3). Mann-Whitney rank sum test analysis found a higher level of TNF-α in the EPA diet group compared with the other groups. Consistent with an increased injury, extravascular lung water significantly increased in the EPA group. However, the permeability was not different among the three groups (Figs. 3 and 4).

No difference was observed in histologic feature between groups. Histologic analysis showed an interstitial infiltration with polymorphonuclear cells, associated with blood vessel congestion and bronchial lysis in all groups after PA infection. No difference, based on the histologic scoring, was observed among the different diets (Table 4).

DISCUSSION

A 3-wk dietary supplementation with various PUFAs before PA lung injury influences the acute inflammatory response and lung functional consequences demonstrated by the accumulation of extravascular water. Using a sublethal bacterial load, the aim of the study was to evaluate the immunomodulatory effect of PUFAs in the early response in severe acute lung injury.

Immunomodulatory effects of PUFA. PUFAs have immunomodulatory properties that previously have been described:

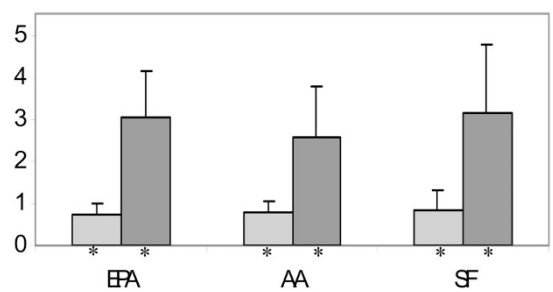

Figure 2. Indexed permeability in infected (n = 10) and noninfected (n = 10) animals. Results are expressed as means and SEM. ☐ Noninfected; ■ Infected. *$p < 0.0001$ *vs* the infected groups.

by down-regulating the inflammatory response, omega 3 PUFAs can be opposed to omega 6 PUFAs, which up-regulate these parameters (1,2). Endres *et al.* (3) studied the influence of n-3 fatty acid dietary supplementation in healthy volunteers. *In vitro*, endotoxin-stimulated peripheral blood mononuclear cells presented a 43% decrease of IL-1β release after 6 wk of supplementation; an additional 18% decrease was observed 10 wk after the end of the supplementation. The production of IL-1α and TNF-α followed the same pattern. Twenty weeks after the end of the supplementation, the production of IL-1β, IL-1α, and TNF-α returned to the presupplemental level. The decreased production of IL-1 and TNF-α was associated with a decreased ratio of AA to EPA in mononuclear cell membrane phospholipids. In a comparable work, Meydani *et al.* (4) showed after 3 mo that n-3 fatty acid supplementation reduced total IL-1β, TNF-α, and IL-6.

In our study, the diets' compositions were designed to provide similar quantities of essential fatty acids but predominance in one PUFA series (Table 2) to appreciate the effect of each fatty acid series while in most of the other studies, PUFA effects are usually analyzed by changing the omega-6 to omega-3 ratio. We found a surprisingly higher TNF-α level in BAL in the EPA diet group compared with the other groups, conflicting with several other studies. This higher level of TNF-α was not associated with any difference in bacterial clearance from the lungs (data not shown). These discrepancies could be related to the injury itself and the inflammatory response generated after PA instillation; in fact, this bacterium is particularly pathogenic with a lot of different virulence mechanisms. Moreover, it has been reported that species differences after lipid diets and the increase that we observed are consistent with other published work in mice (21,22).

PUFA effects on lung permeability. In PA-induced acute lung injury as in our study, alveolar capillary permeability is increased on both epithelial and endothelial barriers (23,24). Using different fat blends, Mancuso *et al.* (9) studied the influence of PUFAs on alveolar capillary permeability in a *Salmonella enteritidis* endotoxin model. In this study, alveolar protein permeability increased and hypotension was reduced with PUFA fat blends that contained more omega 3 fatty acids. To our knowledge, no other study has assessed PUFA effect on lung permeability. In our study, we failed to find any difference in permeability between groups. This could be related to the lack of sensitivity of the methods used to study lung permeability in mice compared with rats.

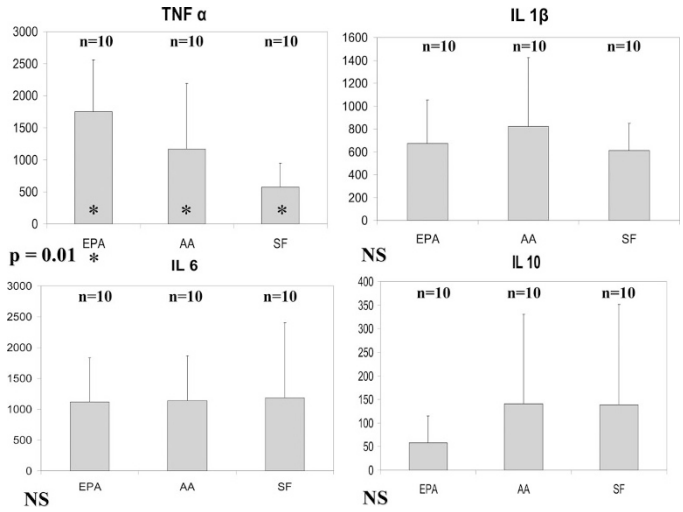

Figure 3. Measurement of cytokines in the BAL in PA-injured mice. Results are expressed as means and SEM. $*p < 0.05$ *vs* the two other groups.

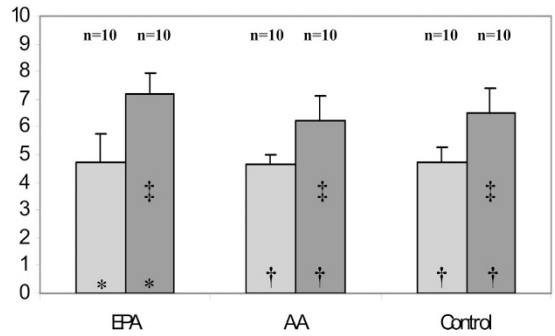

Figure 4. Lung W/D in infected and noninfected animals. Results are expressed as means and SEM. ▒ Noninfected; ■ Infected. $*p = 0.001$; $\dagger p = 0.0001$; $\ddagger p = 0.03$; $*p < 0.001$ *vs* the infected groups.

PUFA effects on lung edema. In an isolated perfused lung model, Koch *et al.* (12) demonstrated that omega-3 fatty acid infusion decreased edema formation after chemical injury. Breil *et al.* (10), in a comparable setting, showed that n-3 lipid infusion had anti-inflammatory effects on lung vasculature. These authors discussed a role of the EPA metabolism with a generation of less potent inflammatory eicosanoids resulting in a reduced edema. Sane *et al.* (11) reported, in a rat endotoxin model, that EPA supplementation depressed AA content and leukotriene B (4) generation in alveolar macrophages as well as plasma thromboxane B (2), leading to decreased pulmonary edema. Omega-3 fatty acids also reduce the release of proinflammatory lipid-derived mediators, consistent with a decrease of lung edema level.

In our study, we found a higher level of edema in the EPA diet group compared with the others. These discrepancies compared with the literature could also be related to the model that we used. In facts bacterial pneumonia induces a strong inflammatory response. The increase of the extravascular lung water that we found may reflect an alteration of the equilibrium between alveolar liquid clearance and pulmonary liquid clearance. A major inflammatory response may also be responsible for this increase; in this hypothesis, EPA could be responsible for an enhancement of the animal inflammatory response, and the increased extravascular lung water could reflect a side effect, consistent with the higher levels of TNF-α in the EPA group.

Table 4. *Histological scoring of PA injured lungs*

Diet group	EPA ($n = 3$)	AA ($n = 3$)	SF ($n = 3$)
Degree of inflammation	2.33	2.33	2.66
Extension	D	D	D

AA, Arachidonic acid diet group; EPA, Eicosapentaenoic acid diet group; SF, control diet group. The scale was 0 to 3 (0: absent, 1: mild, 2: moderate, 3: intense) for inflammation. D, diffuse.

Effect of PUFAs in mortality after bacterial infection. Previous reports showed that fish oil, containing high concentration of omega-3 fatty acids, could prevent mortality in infectious models. This was documented in *Klebsiella pneumoniae* infection (14), *Staphylococcus aureus* and *Bacteroides fragilis* abscesses models (13), and *Klebsiella pneumoniae* and *Plasmodium berghei* infections (15). This parameter was never evaluated in PA acute lung injury. In our study, we did not find any difference in early mortality among the groups. However, our study was not designed to do so, because the animals were killed very early after infection (24 h). It therefore was not possible to assess the consequences on spontaneous mortality rate on long-term follow-up.

In conclusion, our study clearly shows that PUFAs can modulate the inflammatory response. After a PA challenge, we observed major changes in the early phase of the injury, depending on the diet of the animals, EPA being associated with an increase of TNF production as well as lung edema. On this basis, the question of whether this effect on inflammation could be beneficial for the animal should be studied with a long-term assessment of the inflammatory response and the outcome.

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