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Influence of Dietary (n-3)-Polyunsaturated Fatty Acids on Leukotriene B_4 and Prostaglandin E_2 Synthesis and Course of Experimental Tuberculosis in Guinea Pigs

Summary: In the present study eicosanoid synthesis was studied in macrophages of guinea pigs fed different amounts of (n-6)- and (n-3)-polyunsaturated fatty acids (PUFA). Three groups of weanling guinea pigs were fed by isocaloric diets differing only in their contents of PUFA: controls with 2.8 Cal% of linoleic acid (LA; 18:2(n-6)); (n-6)-rich fed animals with 15.4 Cal% of LA; and (n-3)-rich fed animals with 10.1 Cal% of LA, 1.4 Cal% of eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)). After 13 weeks half the number of animals from each group was infected i.m. by 180 colony forming units of *Mycobacterium tuberculosis* strain H37Rv. Seven weeks after infection the release of leukotriene (LT)B_4 and prostaglandin (PG)E_2 was quantified in calcium ionophore stimulated whole blood, peritoneal macrophage cultures and alveolar macrophages by immunoassays after high performance liquid chromatography. Synthesis of LTB_4 and PGE_2 was found to be reduced in (n-3)-rich fed guinea pigs ($p < 0.05$), and equivalent between controls and (n-6)-rich fed animals. Controls and (n-6)-rich fed animals showed the same mycobacterial counts in the spleen whereas (n-3)-rich fed guinea pigs demonstrated an increased number of mycobacteria ($p < 0.05$). Our results demonstrate that an increased dietary intake of (n-3)-PUFA suppress LTB_4 and PGE_2 synthesis. The increased number of *M. tuberculosis* found in the spleens of (n-3)-rich fed animals could represent persistence of the experimental infection. It may be speculated that a functional relationship exists between the two findings.

Introduction

The (n-3)-polyunsaturated fatty acids (PUFA) mainly found in fish oils have been the subject of numerous studies focusing on their protective role against cardiovascular and autoimmune diseases [1, 2]. It has been shown that PUFA of the (n-6)-family may under certain circumstances be proaggregatory, proinflammatory and immunosuppressive [3]. On the other hand, it is believed that PUFA of the (n-3)-family from fish oils can ameliorate these tendencies and exert beneficial effects on symptoms of atherosclerosis, heart disease and inflammation [4, 5]. Recent data indicate that fish oil decreases resistance to infection with *Salmonella typhimurium* [6].

The role of diet, e.g. protein, energy, and vitamins, has been shown to play a major role in the natural resistance against infection with *Mycobacterium tuberculosis* [7–10]. However, the significance of different PUFA has not been studied so far. PUFA of the (n-3) and (n-6) series are precursors of leukotrienes and prostaglandins, which are involved in inflammatory processes [11]. It is established that the production of potent lipoxygenase and cyclooxygenase products such as leukotriene (LT)B_4 and prostaglandin (PG)E_2 is determined by the presence of (n-6)- and (n-3)-PUFA [12–14]. Recent studies demonstrated that (n-3)- and (n-6)-PUFA have the potential to interfere with LTB_4 and PGE_2 formation by interacting with lipoxygenase and/or cyclooxygenase [15, 16]. Therefore, diets providing these PUFA are expected to modify immune responses and inflammatory processes mediated by these eicosanoids.

From epidemiological studies it is known that the incidence of tuberculosis is increased in Eskimos with traditionally high consumption of (n-3)-rich fish oil [17, 18]. Additionally, *in vitro* results have shown that a high (n-3)-PUFA-intake may result in abnormalities in macrophage functions [13, 19]. However, macrophages play a major role in the pathogenesis of infection with *M. tuberculosis*, and LTB_4 as well as PGE_2 are mainly synthesized by these cell types, being involved in several steps of the immune response [20, 21]. In a separately published paper from this study

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Infection 22 (1994) No. 2

Table 1: Fatty acid composition of the different diets fed to guinea pigs.

Fatty acid		Control group (group A)	(n-6)-group (group B)	(n-3)-group (Group C)
		% of calories		
14 : 0		0.08	0.08	0.91
16 : 0		5.79	2.08	3.51
16 : 1		0.06	0.05	1.20
17 : 0		0.05	0.02	0.06
18 : 0		8.50	1.70	1.50
18 : 1	(n-9)	8.08	6.03	4.85
18 : 2	(n-6)	2.83	15.37	10.06
18 : 3	(n-6)	0.00	0.00	0.02
20 : 0		0.25	0.10	0.08
20 : 1	(n-9)	0.02	0.05	0.19
20 : 3	(n-6)	0.00	0.00	0.02
20 : 4	(n-6)	0.00	0.01	0.05
20 : 5	(n-3)	0.00	0.02	1.36
22 : 5	(n-6)	0.01	0.00	0.02
22 : 0		0.08	0.20	0.15
22 : 5	(n-3)	0.00	0.00	0.21
22 : 6	(n-3)	0.01	0.01	0.89

detailed results on the progress and staging of the experimental tuberculosis and the tuberculin tests under these dietetic regimens are given. This paper presents the results of eicosanoid formation in macrophages of guinea pigs fed different amounts of (n-6)- and (n-3)-PUFA with and without infection with *M. tuberculosis.*

Materials and Methods

Animals: Male Duncan-Hartley guinea pigs (2 weeks old) were obtained from a specified pathogen-free colony of the Thomae Laboratory, Biberach, FRG. The animals, with body weights between 200–300 g, were assigned randomly to three groups (the mean body weight was equal for the three groups). These groups received three different diets over the next 20 weeks. All animals were kept in stainless steel cages (one guinea pig per cage) and had free access to food and water. A 12 h light-dark cycle and a temperature of 22°C were maintained in the room. Body weights were recorded twice weekly during the experiment. Animal care was conducted according to the NIH Guide for the Care & Use of Laboratory Animals.

Diets: Synthetic diets were obtained from Unilever (Vlaardingen, The Netherlands). The diets were flushed with nitrogen and stored at – 20°C in the dark. The guinea pigs received freshly thawed diet every day. The animals were fed one of the following adequate isocaloric diets: Group A (control group): 20.8 Cal% cocoa butter, rich in saturated fatty acids with 2.8 Cal% of LA (18:2(n-6)); group B ((n-6)-group): 26.0 Cal% sunflower oil, rich in LA (15.4 Cal%); and group C ((n-3)-group): 11.0 Cal% fish oil, with 10.1 Cal% of LA, 1.4 Cal% of eicosapentaenoic acid (EPA; 20:5(n-3)) and 0.9 Cal% of docosahexaenoic acid (DHA; 22:6(n-3)). The fatty acid composition of the diet administered is given in Table 1. The composition of the diets was analyzed every second week and found to be stable. Besides the fat-free basic diet containing casein (23 Cal%) and minerals, all oils contained sufficient amounts of vitamins including α-tocopherol-acetate. After infection with *M. tuberculosis*, the animals received the same diet as in the period prior to infection.

Infection of guinea pigs: About 13 weeks after initiation of experimental diets, half the number of animals from each group were randomly selected and infected intramuscularly with 180 colony forming units (cfu) of *M. tuberculosis* strain H37Rv (ATCC 35837) in the right thigh. The inocula were prepared according to methods described previously [22, 23]. The remaining uninfected animals acted as controls in that group. Seven weeks after infection, infected and uninfected animals of all groups (n = 17 of each group) were anaesthetized with ketamine (50 mg/kg, Ketavet, Parke-Davis, Munich, FRG, intraperitoneally). Blood sampling, peritoneal- and bronchial lavage were carried out as described below, and the animals were sacrificed by exsanguination. Blood cell counts, haemoglobin, protein, albumin, creatinine etc. were measured by routine laboratory analyses. Numbers of viable mycobacteria in spleens were determined by plating suitable dilutions of individual whole organ homogenates in sterile cold saline medium on Middlebrook 7H10 agar (Difco Laboratories, Inc., Detroit, MI) and counting cfu after incubation for 21 days at 37°C.

Release of LTB_4 and PGE_2 in whole blood: The method used for determining the release of LTB_4 and PGE_2 in guinea pig whole blood was similar to that already described [24]. Briefly, blood was collected from guinea pigs into polypropylene tubes containing heparin (100 U/ml). Blood (0.2 ml) was added to 1.5 ml Eppendorf tubes. Calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO), dissolved in dimethyl-sulfoxide, was added in a concentration of 15 µg/ml and incubation was carried out at 37°C for 20 min. At the end of the incubation, samples were centrifuged (12,000 x g for 2 min), and the cell-free plasma was removed and spiked, 1,000 dpm each, with (^{3}H)LTB_4 (7.0 TBq/mmol) and (^{3}H)PGE_2 (5.8 TBq/mmol), both obtained from Du Pont-New England Nuclear, Boston, MA, as internal standards to correct for recovery losses. Samples were passed through Sep-Pak cartridges containing octadecylsilyl silica (10 x 10 mm; Waters Associates, Milford, MA) that had been prewashed sequentially with 50 ml of each of methanol, water, methanol/water (1:9, vol/vol) containing 14 mM EDTA, pH 7.4, and water. After applying the samples, the columns were rinsed successively with 5 ml of water, 15% (vol/vol) aqueous methanol, and benzene. LTs and PGs were then eluted with 2 ml of absolute methanol. Recovery was found between 84 and 89% as analyzed with ^{3}H-labeled LTB_4 and PGE_2. Aliquots of the methanol fractions were dried in a SpeedVac concentrator (Savant Instruments, Inc., Hicksville, NY) and resuspended in 30% (vol/vol) aqueous methanol. Samples were stored at – 40°C under argon until further purification by HPLC and immunoassay analysis as described below.

Eicosanoid synthesis in peritoneal macrophage culture: Resident peritoneal macrophages were isolated from guinea pigs by lavage with sterile PBS [19]. 1–2 x 10^6 peritoneal macrophages were plated on 5-cm diameter plastic tissue culture dishes (Becton Dickinson & Co., Mountain View, CA) in 3 ml of Eagle's MEM (Boehringer Mannheim Biochemicals, Mannheim, FRG) containing 5% foetal bovine serum for 2 h at 37°C under 5% CO_2/95% air. After removal of nonadherent cells by washing the dishes twice, the adherent cells were placed in 2 ml of serum-free

Eagle's MEM containing 15 µg/ml of calcium ionophore A23187. The cells were then cultured for 2 h at 37°C under 5% CO_2/95% air. More than 95% of the cells were identified as macrophages, and viability as determined by trypan blue exclusion was always greater than 98%. The cell supernatants were removed, spiked with ^{3}H-labeled LTB_4 and PGE_2 as internal standards and passed through activated Sep-Pak C18 cartridges as described above. The subsequent procedure included fractionation by HPLC and quantification by immunoassays.

Isolation and release of eicosanoids by alveolar macrophage culture: Bronchoalveolar lavage and cell analysis was carried out as previously described [25]. Alveolar macrophages obtained by bronchoalveolar lavage were incubated at 37°C in RPMI 1640-medium (Whittaker M. A. Bioproducts, Walkersville, MD) in sterile 24-well culture plates (Falcon Labware, Oxnard, CA). After a 2 h incubation period to permit adherence, the cells were washed three times with iced RPMI-1640, after which calcium ionophore A23187 in a concentration of 15 µg/ml RPMI-1640 was added to the plate. After incubation (1 x 10^6 cells, 3 h, 37°C), the viability of macrophages at the end of the incubation period was > 90% in all cases, as determined by trypan blue exclusion. Supernatants from stimulated alveolar macrophages in RPMI-1640 were spiked with ^{3}H-labeled LTB_4 and PGE_2, passed through activated Sep-Pak C18 cartridges and quantified after HPLC by immunoassays.

High performance liquid chromatography: Reversed-phase HPLC was performed isocratically on a C18-Hypersil column (4.6 x 250 mm, 5-µm particles; Shandon, Runcorn, UK) with a C18 precolumn (Waters Associates, Milford, MA). The mobile phase consisted of 38% (vol/vol) acetonitrile in aqueous buffer (0.1% acetic acid, 1 mM EDTA, pH 5.6, adjusted with ammonium hydroxide). The flow rate was 1 ml/min. A 300-µl aliquot of each 1-ml fraction collected during HPLC was counted for calculation of tritium recovery, and 700 µl were dried for the subsequent immunoassay.

Analysis of LTB_4: LTB_4 was quantified by radioimmunoassay. The LTB_4 antibody was kindly provided by Dr. *A. W. Ford-Hutchinson* (Merck Frosst, Pointe-Claire/Dorval, Quebec, Canada). The assay procedure was a modification of the one described previously [26, 27]. A 700-µl aliquot of the HPLC fractions was evaporated to dryness and resuspended in 100 µl assay buffer (0.9% NaCl, 0.1% gelatin, 10 mM EDTA, 0.1% sodium azide in 10 mM phosphate buffer, pH 7.4). Tubes for LTB_4 standard curve also contained 700 µl dried, LT-free HPLC eluate. Antibody, diluted 1:10^5 in assay buffer, was added to the samples and standards. After mixing and a 30-min preincubation period at room temperature, (^{3}H)LTB_4 (3,000 dpm) was added in 100 µl of assay buffer, mixed, and incubated at 4°C for 16–20 h. Unbound (^{3}H)LTB_4 was removed by addition of 1 ml of ice-cold charcoal suspension (0.63% charcoal, 0.063% dextran in 10 mM phosphate buffer, pH 7.4) and, after 4 min, by centrifugation at 1,400 x g for 15 min at 4°C. The supernatant was added to 10 ml of scintillation fluid for counting of radioactivity. The lower detection limit of the assay system for LTB_4 was 35 fmol/tube. The molar crossreactivities at 50% binding were as follows: LTB_4 100%; cysteinyl LTs including 12(S)-hydroxyeicosatetraenoate < 0.05%; 6-trans-LTB_4 2.0%; 6-trans-12-epi-LTB_4 0.6%; ω-hydroxy-LTB_4 1.0%; ω-carboxy-LTB_4 1.0%; PGE_2 < 0.01%; arachidonic acid < 0.01%; $PGF_{2\alpha}$ < 0.01%.

PGE_2 analysis: PGE_2 was quantified by ELISA. The assay system (Cayman Chemicals, Ann Arbor, MI) was used and EIA was performed according to the provided procedure. The PGE_2 antibody crossreacted with prostanoids as follows: PGE_2 100%; 15-keto PGE_2 9.2%; PGE_1 5.0%; PGA_1 < 0.2%; PGB_1 < 0.2%; PGB_2 < 0.2%; PGD_2 < 0.2%; $PGF_{1\alpha}$ < 0.2%; 6-keto $PGF_{1\alpha}$ < 0.2%; $PGF_{2\alpha}$ < 0.2%; 13, 14-dihydro-15-keto $PGF_{2\alpha}$ < 0.2%; thromboxane B_2 < 0.2%. The lower detection limit for PGE_2 was at 40 fmol/tube.

Statistical analysis: Throughout the experiments, principles of randomization were followed. Statistical Analysis System (SAS Inst., Cary, NC) was used to analyze the data. Multiple regression analysis was used to estimate the statistical coefficients in an interdependent system of linear equations. Student's t-test or the Wilcoxon-Mann-Whitney test was used for statistical comparison. $p < 0.05$ was taken as minimum level of significance.

Results

Nutritional Status and Weight Gain.

There was no effect of consumption of different diets on blood parameters, including serum albumin and haemoglobin concentrations, within and between the three groups of animals. Infection with *M. tuberculosis* also had no effect on these parameters.

The amounts of diet consumed by the animals and the weight gains were similar in all dietary groups at various times of the experiments. There were no significant differences in weight gain between the infected and non-infected animals.

Release of LTB_4.

The release of LTB_4 by stimulated whole blood cells, peritoneal macrophages and alveolar macrophages obtained from infected and non-infected guinea pigs fed different diets is shown in Figure 1A. In all compartments studied the LTB_4 concentrations were significantly decreased in the (n-3)-rich fed group compared to the control group and the (n-6)-rich fed group. The controls and the (n-6)-rich fed group showed similar LTB_4 levels. There were no significant differences in LTB_4 release for all compartments between infected and non-infected animals.

PGE_2 Release.

The capacity to release PGE_2 measured in whole blood, peritoneal and alveolar macrophages obtained from all infected and non-infected animals receiving different diets is presented in Figure 1B. As shown for LTB_4, our findings illustrate that the PGE_2 release was significantly decreased in all studied compartments of (n-3)-rich fed guinea pigs compared to the (n-6)-rich fed and control animals which both showed similar PGE_2 release. With the exception of the PGE_2 release from whole blood, there was no significant difference between infected and non-infected animals for either, peritoneal and alveolar macrophages.

Numbers of Mycobacteria in Spleen.

The number of viable mycobacteria in spleens of infected guinea pigs 7 weeks after infection were (number of organisms/organ, n = 17 animals of each group) 18,391 ± 5,688 (mean ± SEM) in group A, 15,146 ± 4,276 in group B, and

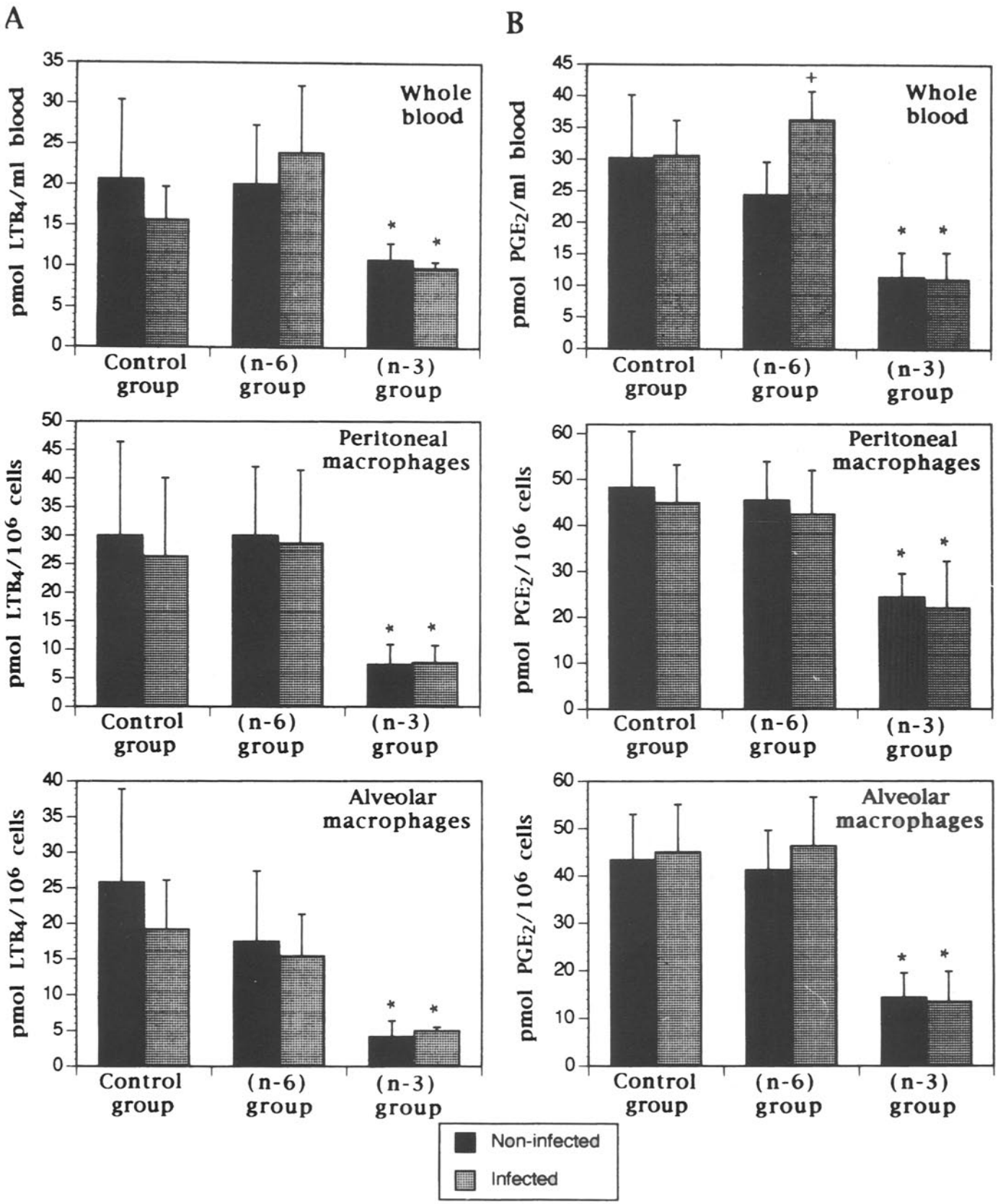

Figure 1: Synthesis of LTB_4 (A) and PGE_2 (B) by ionophore stimulated whole blood, peritoneal macrophages and alveolar macrophages obtained from *Mycobacterium tuberculosis* infected and non-infected guinea pigs fed isocaloric diets differing only in PUFA contents. * $p < 0.05$ versus control and (n-6) group; + $p < 0.05$ versus non-infected (n-6) group.

35,532 ± 8,122 in group C ($p < 0.05$ of group C against group B and against group A).

Discussion

Our results demonstrate that dietary supplementation with (n-3)-PUFA, mainly EPA and DHA which are highly enriched in fish oil, suppresses LTB_4 and PGE_2 synthesis in blood cells, peritoneal and alveolar macrophages. It also influences persistence of the experimental tuberculosis. It is established that endogenous eicosanoid metabolism can be modified by type and amount of fat consumed in the diet [28]. Fish oil supplementation is able to reduce production of PG of the 2 series and of the 4 series *in vitro* and *in vivo* [19]. In our study, a diet rich in (n-3)-PUFA caused an equivalent inhibition of LTB_4 and PGE_2 production in macrophages. In contrast to the (n-3)-rich diet an increased administration of (n-6)-PUFA as LA did not alter eicosanoid generation of macrophages. Infection with *M. tuberculosis per se* had no effect on eicosanoid biosynthesis in blood cells, peritoneal or alveolar macrophages in all different groups of animals. This is in contrast to infection with *Toxoplasma gondii* [29], which leads to diminished synthesis of eicosanoids. We conclude that the effect of dietary (n-3)- and (n-6)-PUFA on eicosanoid formation in guinea pigs is not influenced by experimental tuberculosis.

There have only been a few reports about the influence of different fatty acid supplementation on the course of infection. *Huang* et al. [30] reported that (n-3)-rich fed mice show a higher number of T-cells and macrophages than (n-6)-rich fed animals. Infection with *Listeria monocytogenes*, however, led to a significantly lower number of T-cells and macrophages in the peritoneal exudate of (n-3)-rich fed mice compared to (n-6)-rich fed animals. Peritoneal macrophages obtained from mackerel oil fed rats produced fewer eicosanoids than those from other dietary groups, e.g. sunflower oil or hydrogenated coconut oil [31]. However, infection of these animals via intraperitoneal injection of rat cytomegalovirus resulted in a significantly decreased eicosanoid production in all groups, irrespective of dietary fat type. This demonstrates that this viral infection eradicates the differences in eicosanoid generation of macrophages. This is in contrast to our findings in *M. tuberculosis*-infected guinea pigs.

In our study all animals were sacrificed 20 weeks after initiation of experimental diets. Thus, the significantly higher numbers of viable *M. tuberculosis* found in the spleens of guinea pigs maintained on fish oil supplementation could represent persistence of the experimental infection and reflect a decreased immunoreactivity and enhanced immunosuppression. This immunodysregulation is reflected in the reduced LTB_4 and PGE_2 synthesis. There is evidence of a pathogenetic role for LTB_4 in several diseases characterized by a destructive host inflammatory response, including rheumatoid arthritis, inflammatory bowel disease and cystic fibrosis [32–34]. LTB_4 is known to be involved in the proliferation and activation of T-lymphocytes [35]. Studies have further demonstrated that interfering with the chain of events initiated by LTB_4 results in a decreased inflammatory response and decreased killer cell activity [13, 36]. PGE_2 is also an important regulatory modulator of immunity regarding host defence responses to infection, and is involved in many actions of macrophage functions and mediator synthesis [21]. Moreover, recent studies indicated that lipoxygenase products are important in the generation and maintenance of immune granulomatous inflammatory responses [37].

Acquired resistance against tuberculosis depends on specific T-lymphocyte and mononuclear phagocytes. *M. tuberculosis* is capable of replicating in mononuclear phagocytes, and it is suggested that both helper and cytolytic T-cells participate in the immune response and resistance to tuberculosis [38]. Decreased LTB_4 and PGE_2 synthesis induced by (n-3)-PUFA may derange the subtle coordination of the immune response in tuberculosis leading to persistence of the infection. There is also evidence that cytokines, such as interleukin 1, play a role in the pathogenesis of tuberculosis [39]. Because it is well known that PGs and LTs are also involved in the production of interleukin 1 in macrophages, this interaction might also contribute to a decreased resistance against *M. tuberculosis*. Recently, the inducing role of tumour necrosis factor in the development of bactericidal granulomas during BCG infection in mice has been studied [40]. The exact underlying mechanism warrants further investigation; however, the current studies clearly demonstrate that a fish oil diet rich in EPA and DHA decreases host resistance to experimental tuberculosis. Our results should be regarded in the light of previous epidemiological findings with respect to an increased prevalence of tuberculosis in Eskimos with traditionally high consumption of (n-3)-rich fish oil [17, 18]. Besides beneficial effects for the prevention of atherosclerosis and other diseases, alterations induced by (n-3)-PUFA may be detrimental with regard to the course of *Salmonella* infections [6] and tuberculosis.

Certainly persistence of experimental tuberculosis in guinea pigs which are fed by (n-3)-PUFA enriched food and decreased synthesis of LTB_4 and PGE_2 point towards a possible mutual pathogenic pathway. However, further kinetic studies of infection at different time points and investigations about the pathways of defence have to be carried out to ascertain the significance of these eicosanoids for the resistance against tuberculosis.

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Zusammenfassung: Einfluß einer Diät mit (n-3)-mehrfach ungesättigten Fettsäuren auf die Leukotrien B_4- und Prostaglandin E_2-Synthese und den Verlauf der experimentellen Tuberkulose bei Meerschweinchen. In der vorliegenden Studie wurde die Eikosanoidsynthese in Makrophagen von Meerschweinchen untersucht, die mit unterschiedlichen Gehalten (n-6)- und (n-3)-mehrfach ungesättigten Fettsäuren (PUFA) gefüttert wurden. Drei Gruppen entwöhnter Meerschweinchen wurden mit isokalorischen Diäten gefüttert, die sich nur in ihrem Gehalt an PUFA unterschieden: Kontrollen mit 2,8 Cal% Linolsäure (LA. (18:2(n-6)); Tiere mit (n-6)-angereichertem Futter mit 15,4 Cal% LA und Tiere mit (n-3)-angereichertem Futter mit 10,1 Cal% LA, 1,4 Cal% Eikosapentaensäure (20:5(n-3)) und 0,9 Cal% Dokosahexaensäure (22:6(n-3)). Nach 13 Wochen wurde die Hälfte der Tiere aus jeder Gruppe mit 180 Kolonie-bildenden Einheiten des Stammes *Mycobacterium tuberculosis* H37Rv i.m. infiziert. Sieben Wochen nach Infektion wurde die Freisetzung von LTB_4 und PGE_2 in Kalzium-Ionophor stimuliertem Vollblut, Peritonealmakrophagen und Alveolarmakrophagen mittels Immunoassays nach Hochdruckflüssigkeitschromatographie quantifiziert. Die Synthese von LTB_4 und PGE_2 war reduziert bei den (n-3)-reich gefütterten Meerschweinchen ($p < 0,05$) und äquivalent bei den Kontrollen und (n-6)-reich gefütterten Tieren. Kontrolltiere und (n-6)-reich gefütterte Meerschweinchen wiesen die gleiche Zahl von Mykobakterien in der Milz auf, während sich bei den (n-3)-gefütterten Tieren eine erhöhte Zahl von Mykobakterien zeigte ($p < 0,05$). Unsere Ergebnisse zeigen, daß eine höhere diätetische Zufuhr von (n-3)-PUFA die Synthese von LTB_4 und PGE_2 unterdrückt. Die erhöhte Zahl von *M. tuberculosis* in den Milzen von (n-3)-gefütterten Tieren spricht für eine Persistenz der experimentellen Tuberkulose unter diesen Bedingungen. Möglicherweise existiert zwischen beiden Befunden ein funktioneller Zusammenhang.

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