Influence of n-6 and n-3 Polyunsaturated Fatty Acids on the Resistance to Experimental Tuberculosis

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It has previously been shown that the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)) possess antiinflammatory properties and can interfere with immune functions. To evaluate whether this would affect resistance to infection, we studied the influence of different types of fatty acids (FAs) on experimental tuberculosis in an animal model. Three groups of 26 weanling guinea pigs were fed isocaloric diets with 26 cal% fat that differed in FA composition with respect to saturated FAs, linoleic acid (18:2(n-6)), eicosapentaenoic acid (20:5(n-3)), and docosanexaenoic acid (22:6(n-3)) as follows: (1) reference (REF) group: 14.8 cal% saturated FAs and 2.8 cal% linoleic acid; (2) n-6 group: 4.6 cal% saturated FAs and 15.4 cal% linoleic acid; (3) n-3 group: 6.3 cal% saturated FAs, 10 cal% linoleic acid, 1.4 cal% eicosapentaenoic acid, and 0.9 cal% docosahexaenoic acid. After 13 weeks, 18 animals from each group were intramuscularly injected with 180 colony-forming units (CFU) *Mycobacterium tuberculosis* strain H37Rv. Eight noninfected animals per group served as controls. Seven weeks later, the mean number of mycobacteria recovered from the spleens of the n-3 group (log 4.34 CFU, standard error of the mean [SEM], 0.12) was significantly higher than from the REF group (log 3.90 CFU; SEM, 0.15) and the n-6 group (log 3.93 CFU; SEM, 0.13; $P < .05$). In addition, the Root Index of Virulence (RIV) showed the most pronounced progression of the disease in the n-3 group. The mean size of the tuberculin reaction was larger in the n-3 group than in the other groups ($P < .05$). There was no significant difference between the n-6 group and the REF group. We conclude that supplementing the diet with n-3 FAs eicosapentaenoic acid and docosahexaenoic acid can affect resistance to *M tuberculosis*, whereas supplementing with n-6 FAs does not.

PREVIOUS STUDIES have demonstrated that polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series, especially eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)), have the potential to alter lymphocyte, monocyte, and macrophage functions.[1-6] Our goal was to evaluate whether dietary n-3 or n-6 fatty acids (FAs) would affect the course of experimental tuberculosis in guinea pigs. *Mycobacterium tuberculosis* was chosen as the infectious agent because (1) its outcome largely depends on the combined action of T lymphocytes and macrophages and (2) it is well known that the progression of tuberculosis is influenced by the nutritional status of the host.[7-11] The guinea pig was selected as the experimental animal because, of all susceptible species, its PUFA metabolism, especially the activity of the enzyme Δ5-desaturase, is closest to the human.[12]

MATERIALS AND METHODS

Study Design and Animal Care

Seventy-eight male weanling Duncan Hartley guinea pigs free from specific pathogens were obtained from a local breeder (Thomae, Biberach, Germany). The weight of the animals at the time of arrival was 252 ± 20 g (mean ± SD). All guinea pigs were kept singly in stainless steel cages with free access to food and water. Immediately after arrival, the animals were randomly assigned to three feeding groups: reference (REF), n-6, and n-3. After a 2-week adjustment period, the experimental diets were started, and after a further 13 weeks, an interval considered long enough to establish a steady state between FAs in plasma and cell membranes, 18 randomly chosen animals from each group were infected with *M tuberculosis*.[2,13] Eight noninfected animals from each dietary group served as controls. The different diets continued until the experiment was terminated 7 weeks later. Throughout the experiment, the animals were weighed three to seven times per week and the amount of food consumed was calculated. Animal care followed the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. The study protocol was approved by the Animal Defence Committee for Laboratory Experiments of the Federal State of Baden-Wuerttemberg.

Diets

The diets consisted of regular guinea pig chow and contained approximately 26 cal% (10% weight) fat with different relative amounts of n-3, n-6, and saturated FAs. The fats were a mixture of sunflower oil, fish oil, and cocoa butter. The composition of the diets and the vitamin and mineral supplements are shown in Table 1. The FA composition of the diets was analyzed once every 2 weeks in our laboratory. The relative amounts of different FAs (average results from five determinations in our laboratory) are shown in Table 2. Vitamin E was added according to the double bonds of the FAs within the fat mixtures (Table 1). All food was prepared in pellet form (Unilever, Vlaardingen, The Netherlands) every 14 days, flushed with liquid nitrogen to prevent autoxidation, and stored at −20°C until the day of consumption. The guinea pigs received fresh thawed pellets every day.

Infection

The infectious dose per animal was 180 colony-forming units (CFU) of the strain H37Rv of *M tuberculosis*. Inocula were prepared according to methods described previously.[14,15] The properties of the strain had been evaluated for infection via the respiratory and subcutaneous routes.[16] The bacteria were administered intramuscularly in a shaved region of the right thigh with 0.5 mL of the suspension. We used a 22-gauge, 1-in needle shielded with a plastic catheter so that no more than 7 mm of the tip was exposed. At the end of the study, the animals were anesthetized intraperitoneally with ketamin (50 to 250 mg,

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Table 1. Composition of the Guinea Pig Diets

Component	g/MJ	Weight%	Cal%
Calcium caseinate (15.7 kJ/kg)	14.64	20.7	23
Vitamin mixture	0.30	0.4	
Mineral mixture	4.76	6.7	
Cellulose	6.50	9.2	
Fat mixture	7.00	9.9	26
Starch (Maizena; 13.65 kJ/g)	37.40	52.9	51
DL-Methionine	0.13	0.2	
Total	70.73	100.0	100

Vitamin E (in addition to the vitamin mixtures and the ingredients of the fats)

Group	mg/MJ	mg/kg Diet
REF	8.1	115
n-6	3.2	45
n-3	5.3	75

Ketavet; Parke-Davis, Munich, Germany) and subsequently exsanguinated by cardiac puncture.

Bacterial Enumeration

Bacterial enumeration of the spleen was performed according to standard methods.[14] The organ was excised aseptically, homogenized with a stomacher,[17] and cultured in triplicate in a blind fashion quantitatively for mycobacteria on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI) at 37°C. The number of CFU was determined after incubation for 14 and 21 days and expressed as $\log_{10}$. The mean difference between three plates from each animal was less than 10%.

Root Index of Virulence

The site of injection, draining lymph nodes, lungs, liver, and spleen were evaluated for the extent of gross disease, and the Root Index of Virulence (RIV) was calculated according to the method of Mitchison et al.[18]

Table 2. FA Composition of the Fat Mixtures

	FEF Group		n-6 Group		n-3 Group	
FA	Weight%*	Cal%†	Weight%*	Cal%†	Weight%*	Cal%†
14:0	0.30	0.08	0.29	0.08	3.49	0.91
16:0	22.27	5.79	8.02	2.08	13.51	3.51
16:1	0.25	0.06	0.21	0.05	4.61	1.2
17:0	0.21	0.05	0.08	0.02	0.23	0.06
18:0	32.71	8.50	6.36	1.7	5.66	1.5
18:1(n-9)	31.08	8.08	23.22	6.03	18.67	4.85
18:2(n-6)	10.90	2.83	59.13	15.37	38.69	10.06
18:3(n-6)	0.00	0.00	0.02	0.00	0.07	0.02
20:0	1.00	0.25	0.38	0.10	0.29	0.08
20:1(n-9)	0.06	0.02	0.18	0.05	0.74	0.19
20:3(n-6)	0.00	0.00	0.00	0.00	0.07	0.02
20:4(n-6)	0.01	0.00	0.04	0.01	0.24	0.05
20:5(n-3)	0.00	0.00	0.08	0.02	5.24	1.36
22:4(n-6)	0.00	0.00	0.00	0.00	0.02	0.00
22:5(n-6)	0.04	0.01	0.00	0.00	0.06	0.02
22:0	0.31	0.08	0.78	0.20	0.55	0.15
22:5(n-3)	0.00	0.00	0.02	0.00	0.82	0.21
22:6(n-3)	0.03	0.01	0.03	0.01	3.44	0.89
PUFAs	11.23	2.94	59.59	15.49	49.59	12.89
P/S ratio		0.20		3.69		2.05

Abbreviation: P/S, polyunsaturated to saturated.
*In relation to the fat mixture.
†In relation to the complete food.

Tuberculin Skin Tests

The animals underwent skin tests intracutaneously with 10 TU purified protein derivative, standard ([PPD-S] Behringwerke, Marburg, Germany) four times: 1 week before infection and on days 14, 25, and 48 after infection. The tests were performed according to standard recommendations on a different shaved area of the back for each injection. The greatest diameter of swelling and reddening was expressed in millimeters. The average of measurements 24 and 48 hours after the application of tuberculin was used for further calculations. In addition, the occurrence of necroses was noted. Skin tests before the infection and in the noninfected group were negative.

Blood Tests and FA Analysis

Blood cell counts and serum protein, albumin, and creatinine were determined by routine methods. Blood for FA analyses was collected in heparinized syringes. Plasma phospholipids (PLs) and cholesterol esters (CEs) were separated by unidimensional thin-layer chromatography according to methods reported previously.[19] After transesterification, FAs were determined by capillary gas chromatography. Values were calculated and expressed as a percentage of the weight of all FAs.

Statistical Analysis

The experiments were performed according to the principles of blind experimentation. The mean number of tubercle bacilli recovered from the spleen of the guinea pigs, the RIV, skin test results, and FA determinations were subjected to variance analysis using the General Linear Models function and the Student *t* test, wherever applicable.[20] Where a distribution was nonparametric, Kruskal-Wallis, Mann-Whitney, or Wilcoxon tests were used. Correlations were calculated using Spearman coefficients. The mean, median, standard deviation of the mean (SD), standard error of the mean (SEM), third quartile (Q_3), first quartile (Q_1), or interquartile range (Q_3-Q_1), respectively, were calculated. However, to allow comparison with previous studies, results for plasma FAs are shown as the mean ± SD.

RESULTS

Nutritional Status and Somatic Data

The amount of food consumed and the corresponding weight gain were similar in all dietary groups throughout the experiment (Fig 1). Different diets had no effect on serum albumin, hemoglobin, and creatinine concentrations (results not shown). The relative liver weight (as a percentage of total body weight) for infected animals was highest in the n-3 group ($P < .01$ for differences among all groups; Fig 2). Furthermore, for infected animals, there were significant differences for the relative spleen weight between the n-3 group and the REF group ($P < .01$). Among noninfected animals, spleen weight was highest in the n-6 group (median, 1.09) and lowest in the REF group (median, 0.77; $P < .05$). One animal from each group died 1 to 3 weeks after infection with *M tuberculosis* without evidence of disease at autopsy.

FA Analyses

In both plasma lipid fractions (CE and PL), the n-6 group had the highest linoleic acid level and the n-3 group the highest eicosapentaenoic acid level (Table 3). The FA composition of plasma CE was closely correlated with the composition of plasma PL. Dihomogammalinolenic acid (20:3(n-6)) was re-

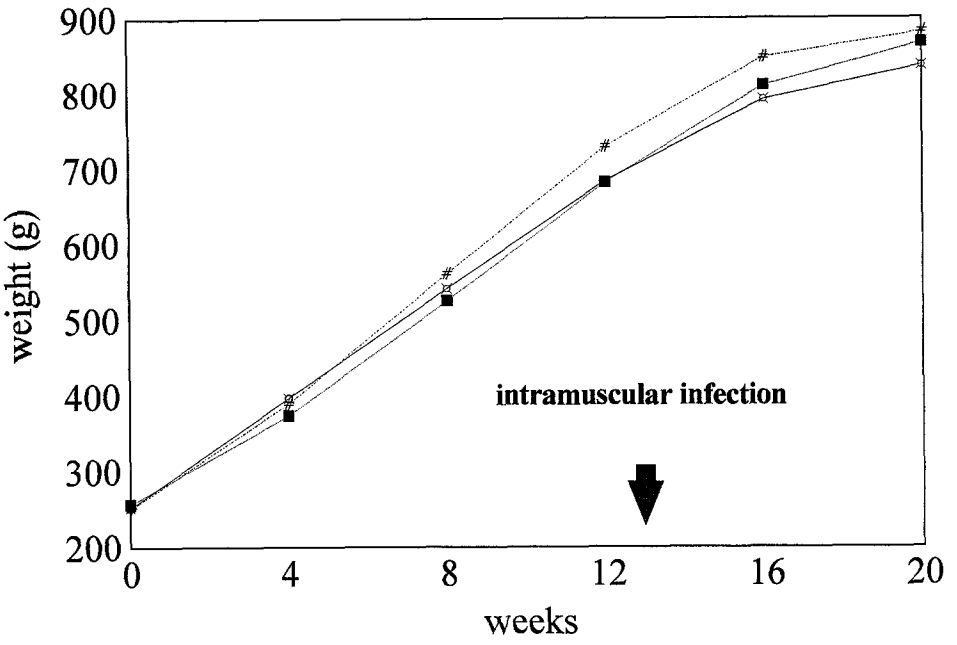

Fig 1. Mean weight gain in animals infected with *M tuberculosis*. (¤) REF group; (■) n-6 group; (#) n-3 group (n = 17 per group, mean ± SEM). *$P < .05$.

duced in PL of infected animals compared with noninfected animals ($P < .05$). Arachidonic acid (20:4(n-6)) did not differ significantly in plasma PL between the REF and n-6 group, and its content was highest in the n-3 group (Table 3).

Bacterial Enumeration in the Spleen

Seven weeks after infection, the spleen of animals in the n-3 group contained almost twice as many mycobacteria as in the two other groups (Fig 3). The means were log 4.34 for the n-3 group, log 3.90 for the REF group, and log 3.93 for the n-6 group. Differences between the REF group and n-3 group and between the n-6 and n-3 groups were statistically significant ($P < .05$).

RIV

The RIV showed that the course of macroscopic disease was most severe in the n-3 group; the least severe alterations were detected in the REF group (Table 4). The differences between the n-3 and REF groups are highly significant ($P < .01$).

Tuberculin Skin Reactivity

Fourteen and 25 days after infection, there were no differences between the groups. In the last test series performed 48 hours before the death of the animals, the reaction was stronger for animals in the n-3 group than in the n-6 group or the REF group ($P < .05$). Furthermore, the occurrence of necroses in the last test was significantly higher in the n-3 group compared with the n-6 group and the REF group ($P < .05$) (Table 5).

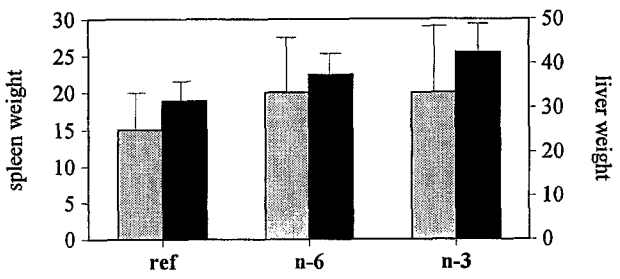

Fig 2. Relative weight (as a percentage of body weight) of liver (■, $\times 10^{-1}$) and spleen (▨, $\times 10^{-2}$) of infected animals (same animals as in Fig 1, mean ± SD).

DISCUSSION

The present study demonstrates for the first time that the course of experimental tuberculosis is more severe in guinea pigs with n-3 FAs in the diets than in animals with diets containing mainly saturated or n-6 FAs. The n-3 FAs eicosapentaenoic acid and docosahexaenoic acid are presently being investigated in the management of a number of inflammatory diseases.[21,22] As a consequence of this study, their impact on resistance to infection should not be overlooked.[23,24] When the effects of dietary n-3 FAs on immunoreactivity are evaluated, a precise definition of the model is mandatory with respect to the species observed, amount and composition of FAs used, somatic data from the animals, FA composition of relevant tissues or plasma, and indices of effector-cell functions.[1,3,4,12,25]

We chose the guinea pig because, of all susceptible species, its polyunsaturated FA metabolism, especially the activity of the enzyme Δ5-desaturase, is closest to human metabolism.[12] In our model, despite the long feeding period of 21 weeks, malnutrition can be ruled out in all groups as a confounding factor raising susceptibility to infection with *M tuberculosis*.[8] On the other hand, the liver and spleen of guinea pigs with a high proportion of unsaturated FAs were heavier than those in the group with mostly saturated FAs. This phenomenon has been described in other species.[1,26] Had we relied exclusively on a score based on the macroscopic appearance of the organs to measure the spread of infection, the results would have been misleading.[18]

The plasma FA determination showed that all feeding groups had sufficient amounts of the essential linoleic acid in the diet. This is important, because a deficiency of linoleic acid has been

Table 3. Plasma FAs in the REF, n-6, and n-3 Groups (mean ± SD)

FA	Plasma PLs			Plasma CEs		
	Infected (n = 17)	Noninfected (n = 8)	*P*	Infected (n = 17)	Noninfected (n = 8)	*P*
REF						
18:1(n-9)	18.8 ± 1.98	16.9 ± 0.73		26.6 ± 2.23	25.4 ± 1.61	
18:2(n-6)	25.1 ± 1.73	25.5 ± 2.02		48.7 ± 1.80	50.1 ± 5.16	
20:3(n-6)	0.27 ± 0.06	0.33 ± 0.08	<.05	0.04 ± 0.07	0.05 ± 0.04	
20:4(n-6)	1.98 ± 0.59	2.31 ± 0.45		0.69 ± 0.23	0.75 ± 0.13	
18:3(n-3)	0.13 ± 0.07	0.14 ± 0.05		0.29 ± 0.17	0.32 ± 0.16	
20:5(n-3)	0.05 ± 0.06	0.03 ± 0.06		ND	ND	
22:6(n-3)	0.02 ± 0.03	0.11 ± 0.15		ND	ND	
Total n-6	28.0 ± 1.91	28.8 ± 2.03		49.8 ± 1.89	51.3 ± 5.01	
Total n-3	0.21 ± 0.11	0.32 ± 0.22		0.31 ± 0.18	0.32 ± 0.16	
Total PUFAs	28.3 ± 1.90	29.8 ± 2.09		50.1 ± 1.90	51.6 ± 4.98	
P/S ratio	0.55 ± 0.05	0.56 ± 0.06		2.45 ± 0.27	2.69 ± 0.89	
n-6						
18:1(n-9)	6.24 ± 0.48	5.75 ± 0.46		7.92 ± 1.57	7.42 ± 1.03	
18:2(n-6)	39.1 ± 1.28	38.3 ± 2.34		69.2 ± 4.64	71.6 ± 2.90	
20:3(n-6)	0.15 ± 0.04	0.27 ± 0.04	<.05	0.05 ± 0.05	0.08 ± 0.08	
20:4(n-6)	2.12 ± 0.37	2.38 ± 0.55		0.58 ± 0.13	0.62 ± 0.16	
18:3(n-3)	0.08 ± 0.04	0.08 ± 0.02		0.13 ± 0.07	0.11 ± 0.04	
20:5(n-3)	0.01 ± 0.03	0.02 ± 0.03		0.02 ± 0.05	0.01 ± 0.01	
22:6(n-3)	0.01 ± 0.03	0.01 ± 0.02		ND	ND	
Total n-6	42.6 ± 1.38	42.5 ± 1.76		70.2 ± 4.68	72.6 ± 2.81	
Total n-3	0.11 ± 0.07	0.11 ± 0.05		0.16 ± 0.08	0.12 ± 0.05	
Total PUFAs	42.7 ± 1.36	42.6 ± 1.77		70.4 ± 4.64	72.7 ± 2.78	
P/S ratio	0.87 ± 0.05	0.86 ± 0.07		3.66 ± 0.83	4.08 ± 0.62	
n-3*						
18:1(n-9)	4.64 ± 0.64	4.63 ± 0.25		6.48 ± 0.78	6.87 ± 0.84	
18:2(n-6)	23.5 ± 2.22	22.3 ± 1.97		54.1 ± 3.66	54.5 ± 5.86	
20:3(n-6)	0.22 ± 0.06	0.31 ± 0.06	<.05	0.11 ± 0.66	0.09 ± 0.09	
20:4(n-6)	4.18 ± 0.88	5.16 ± 0.78	<.05	1.67 ± 0.29	2.03 ± 0.42	
18:3(n-3)	0.14 ± 0.06	0.15 ± 0.04		0.18 ± 0.09	0.15 ± 0.13	
20:5(n-3)	2.70 ± 1.14	3.27 ± 1.91		2.39 ± 1.12	2.58 ± 1.61	
22:6(n-3)	6.23 ± 1.88	7.68 ± 1.07		0.87 ± 0.39	0.65 ± 1.50	
Total n-6	28.5 ± 1.92	28.4 ± 2.06		56.5 ± 3.62	57.1 ± 5.34	
Total n-3	10.2 ± 2.17	12.5 ± 2.06	<.01	3.74 ± 1.55	3.49 ± 2.88	
Total PUFAs	38.6 ± 1.50	40.9 ± 1.04	<.01	60.2 ± 3.05	60.5 ± 2.89	
P/S ratio	0.74 ± 0.05	0.83 ± 0.03	<.01	2.15 ± 0.34	2.22 ± 0.30	

NOTE. *P* values indicate a significant difference for the n-3 *v* n-6 group and the n-3 *v* REF group.
*Noninfected group n = 7.

identified as a risk factor for dermal infections.[27] We could further observe that the proportions of dihomogammalinoleic acid and arachidonic acid were reduced in infected animals compared with noninfected animals of the same group. Although this was statistically significant only in the n-3 group, we would interpret it as an inhibition of the enzyme Δ6-desaturase, which is known to occur in a number of devastating states.[28,29]

The proportions of eicosapentaenoic acid and docosahexaenoic acid were significantly higher in the plasma of the n-3 group than in the other groups. Furthermore, arachidonic acid was more concentrated in the plasma of animals in the n-3 group. We can draw only speculative conclusions: as high and low linoleic acid uptake leads to low arachidonic acid levels, the amount of linoleic acid in the n-3 group may represent the optimum intake.[30] Despite the relatively high proportion of arachidonic acid in the plasma, leukotriene B_4 release from

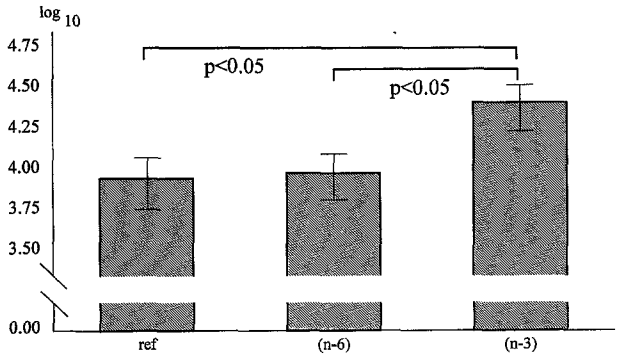

Fig 3. Enumeration of *M tuberculosis* in the spleen of guinea pigs fed diets differing in FA composition (same animals as in Fig 1).

Table 4. RIV

Group	No.	Median	Q3-Q1	Mean ± SD	Maximum	Minimum
REF	17	0.59	0.08	0.63 ± 0.07	0.79	0.49
n-6	17	0.67	0.1	0.66 ± 0.09	0.8	0.39
n-3	17	0.73	0.18	0.75 ± 0.1	0.91	0.59

NOTE. n-3 *v* REF group, $P < .001$; n-3 *v* n-6 group, $P < .05$; n-6 *v* REF group, $P < .05$.

Table 5. Skin Reaction (mean ± SD of the largest diameter 24 and 48 hours after application of 10 TU PPD-S) on Days 14, 25, and 48 After Infection and Occurrence of Necroses

Group	Largest Diameter (mm)			Necroses Day 48 (rel %)*
	Day 14	Day 25	Day 48	
REF	10.74 ± 2.80	18.38 ± 3.74	19.12 ± 3.86	18
n-6	10.47 ± 3.08	18.41 ± 4.12	20.47 ± 5.14	24
n-3	10.12 ± 3.05	19.29 ± 5.25	23.60 ± 4.32†	53‡

*As a percentage of animals tested.
†n-3 *v* REF and *v* n-6, $P < .05$.
‡n-3 *v* n-6, $P < .05$.

alveolar macrophages was reduced in the n-3 group compared with both other groups.[31]

We could not observe a difference between bacterial counts in the spleen of animals with dietary linoleic acid contents of 2.8% and 15.4%. This lack of response to increased dietary n-6 FAs clearly demonstrates that the uptake of PUFAs per se does not increase the susceptibility to infection with *M tuberculosis.* In contrast to linoleic acid, uptake of as little as 2.3% of the n-3 FAs eicosapentaenoic acid and docosahexaenoic acid in the diet leads to impaired resistance to *M tuberculosis.*

Delayed-type hypersensitivity to tuberculin was not reduced in the infected animals of the n-3 group in our experiments. Since delayed hypersensitivity to PPD is reduced in protein-deficient guinea pigs with severe infection with *M tuberculosis,* this suggests that different mechanisms of immunosuppression are involved in the two models.[8]

What is the mechanism by which n-3 FAs create immunosuppression? In vitro, oxidized forms of PUFAs possess antimicrobial effects.[32] This is obviously outweighed by the incorporation of eicosapentaenoic acid and docosahexaenoic acid into the membranes of leukocytes in vivo: it has previously been demonstrated that several lymphocyte and macrophage functions are altered by n-3 FAs.[1-3,6,33] Guinea pig neutrophil granulocytes show a reduced activation response (formation of superoxide anion in response to formyl-methionyl leucyl-phenylalanine [FMLP] and phorbol-myristate-acetate [PMA]) after dietary supplementation with eicosapentaenoic acid.[13] Furthermore, lymphokine-induced activities of mural splenic natural killer cells are attenuated by n-3 PUFAs.[1] Another possible explanation for the reduced activity of granulocytes, macrophages, and lymphocytes induced by eicosapentaenoic acid and docosahexaenoic acid lies in the production of less active inflammatory mediators by mononuclear cells involved in granuloma formation.[2,31,34,35]

One could speculate that a key event leading to the higher load of *M tuberculosis* in the spleen in the group fed with n-3 FAs is the impairment of intracellular killing of mycobacteria.[36] Under the influence of eicosapentaenoic acid and docosahexaenoic acid, a dramatic reduction in the release of lysosomal enzymes, which contribute to this process, has been described.[5]

In summary, our study indicates that supplementing the diet with eicosapentaenoic acid and docosahexaenoic acid impairs the resistance to infection with *M tuberculosis.* The same properties of these n-3 FAs that ameliorate the course of autoimmune diseases may affect immune reactions involved in host defense. The underlying causes remain to be investigated thoroughly.

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