INHIBITION OF \textit{PLASMODIUM BERGHEI} LIVER SCHIZONT DEVELOPMENT AND REDUCTION OF CYTOKINE PRODUCTION CAPACITY IN RATS BY DIETARY FISH OIL SUPPLEMENTATION

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Abstract. Experimental primary infection with \textit{Plasmodium berghei} in rats is known to be influenced by several cytokines. Dietary supplementation of n-3 fatty acids has been shown to influence cytokine production capacity and to protect mice from cerebral malaria. We investigated the effect of dietary fish oil (FO) supplementation on cytokine and nitric oxide production and liver schizont development in male brown Norway rats. Control groups were fed either a corn oil-supplemented diet (CO) or standard lab chow (LC). After six weeks on either diet, rats given supplementary FO had a significantly lower production of interleukin-1 (IL-1) and IL-6 after stimulation with lipopolysaccharide, and also had significantly lower numbers of liver schizonts compared with CO- or LC-fed animals. We conclude that in rats, an FO-supplemented diet reduces the production capacity of IL-1 and IL-6 and inhibits schizont development after intravenous inoculation of \textit{P. berghei} sporozoites. Fish oil did not influence nitric oxide production by peritoneal macrophages.

The development of liver schizonts of \textit{Plasmodium berghei} in rodent hepatocytes is known to be influenced by cytokines. In vitro, interferon gamma, tumor necrosis factor alpha (TNF-\alpha), interleukin-1 (IL-1), and IL-6 have been shown to inhibit both sporozoite penetration and schizont development.\cite{1-4} This inhibition seems to be due to induction of a defensive response in hepatocytes, since preincubation of sporozoites with IL-6 has no influence on schizont development.\cite{4}

Dietary fish oil (FO) supplementation is known to influence cytokine production in several species.\cite{5-7} At the same time, it has been shown to exert a beneficial effect on the course of infectious diseases, such as Gram-negative sepsis in humans and cerebral malaria (in mice).\cite{7} We investigated whether FO supplementation had an influence on schizont development in rats, and whether this influence was accompanied by changes in cytokine production capacity. Nussler and others demonstrated the inducibility of nitric oxide by cytokines and also that nitric oxide strongly inhibits schizont development in vitro.\cite{8,9} Therefore, we assessed the synthesis of nitric oxide by peritoneal macrophages from FO-supplemented and control rats.

MATERIALS AND METHODS

Rats. Male brown Norway rats were obtained from Harlan Olac Ltd., Blackthorn, Bicester, United Kingdom. Rats were housed in specific pathogen-free conditions. At the start of the diets, the rats were five weeks old.

Diets. Three groups of five rats each were fed a fat-free standard reference diet as a dry powder (Purified Diet; Hope Farms, Woerden, The Netherlands) supplemented with 1) 14% (w/w) FO (EPAX 3000 TG; kindly provided by Pronova Biocare A. S., Sandefjord, Norway) and 1% corn oil (to prevent essential fatty acid deficiency i.e., linoleic acid in the FO group) (Maizola CPC, Benelux, Hilversum, The Netherlands) (FO group), 2) 15% corn oil (corn oil group [CO]), and 3) a standard lab chow without supplementation (control group [LC]) (only in experiment 2). In these experiments, oil supplements represented about 28% of the total calorie intake. To overcome the p-aminobenzoic acid (PABA) deficiency of the standard reference diet, PABA was added to the drinking water.

Production of \textit{P. berghei} sporozoites. Sporozoites of \textit{P. berghei} (ANKA strain) were harvested from \textit{Anopheles gambiae} mosquitoes 21 days after infection and purified on a biphasic gradient containing urografin and fetal calf serum (FCS). Live sporozoites in 1 ml of medium 199 were injected into the tail vein of the rats.

Evaluation of the number of schizonts. Forty-four hours after injection of sporozoites, rats were anesthetized with ether and killed by cervical dislocation and livers were removed. Sections of the left liver lobe were fixed in formaldehyde. Paraffin-embedded sections (7 \mu m) were prepared and stained with hematoxylin and eosin. The number of liver schizonts (exo-erythrocytic forms [EEF]) per \textmu m$^2$ of liver section from each rat was assessed by light microscopy.

In vitro stimulation of peritoneal macrophages. After six weeks on either diet (FO, CO, or LC), all rats in each group were anesthetized with ether and killed by cervical dislocation. The peritoneal cavity of each rat was rinsed with 30 ml of sterile phosphate-buffered saline containing 5% FCS. The aspirate was centrifuged and resuspended in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS, 50 \mu M \beta-mercaptoethanol (all obtained from Gibco, Grand Island, NY), and 40 \mu g/ml of gentamicin (Essex, Amstelveen, The Netherlands). The proportion of macrophages in each suspension was assessed by peroxidase staining. From each cell suspension, 2 \times 0.5 ml (all suspensions were cultured in duplicate) was seeded into 24-well culture plates (Costar, Cambridge, MA) at a density of 10$^6$ cells/ml. After overnight culture, supernatants were removed and fresh medium with or without lipopolysaccharide (LPS, serotype 011:B4; Sigma, Brussels, Belgium) (10 ng/ml), was added to each well. The cells were incubated for an additional 24 hr and supernatants were stored at -20°C until
measurement of cytokine and nitric oxide levels. After aspiration of the supernatants, fresh medium was added to the cells, which were then lysed by freezing and thawing. In these lysed cell suspensions, we measured cell-associated IL-1 concentrations.

**Measurement of IL-1.** A cloned murine T cell line (D10.G4.1) was derived from primed lymph node T cells from AKR/J mice. A subline of these cells (D10(N4)M) can be propagated in vitro in the presence of EL4-conditioned medium. Cells were cultured in IMDM supplemented with 5% FCS, 5 × 10⁻⁵ M β-mercaptoethanol, penicillin, streptomycin, and 10% EL4-conditioned medium. For the assay, cells were harvested by centrifugation, washed once, and used at a concentration of 10⁵ cells/200 μl in flat-bottomed wells of microtiter plates in the presence of a suboptimal concentration of 300 U/ml of recombinant human IL-2 (rhIL-2) (kindly provided by Hoffman-La Roche, Nutley, NJ). Cells were labeled with 37 kBq of ³H-thymidine (specific activity = 0.7–1.1 × 10⁶ MBq/mmol; Amersham International, Buckinghamshire, Amersham, United Kingdom) during the last 4 hr of a 72-hr culture period. This assay measures IL-1α and IL-1β. Recombinant human IL-1β (kindly provided by Hoffman-La Roche) was used as a standard. The sensitivity of this assay was 1 pg/ml. All cultures were performed in triplicate and the standard deviation was always < 10%.

The EL4 cells (a murine thymoma-derived cell line) were grown in IMDM supplemented with 5% FCS, 5 × 10⁻⁵ M β-mercaptoethanol, penicillin, and streptomycin. To prepare EL4-conditioned medium, EL4 cells were harvested by centrifugation and incubated at a concentration of 10⁶ cells/ml in large culture flasks in the same medium, together with 10 μg/ml of concanavalin A and 10 ng/ml of phorbol myristate acetate. After 18 hr, the supernatant was harvested and stored at −20°C until use.

**B9 bioassay for IL-6.** Interleukin-6 levels were measured in supernatants by the B9 bioassay. Briefly, the IL-6-dependent murine hybridoma cell line (B9) was grown in IMDM supplemented with 10% FCS, 50 μM β-mercaptoethanol, 40 μg/ml of gentamicin, and 8 U/ml of rhIL-6 at a cell density of 0.1–1 × 10⁵/ml. Before the assay, the B9 cells were harvested by centrifugation and washed twice with IL-6-free medium, then seeded at a concentration of 5,000 cells/200 μl in flat-bottom wells of microtiter plates (Costar Europe, Badhoevedorp, The Netherlands) in the presence of supernatant samples in appropriate dilutions. Proliferation was measured by a pulse with ³H-thymidine during the last 16 hr of an 96-hr culture period, with 37 kBq added per well. Samples were tested in triplicate and always related to a standard curve included in each experiment. One U/ml gave rise to half-maximal proliferation by definition. The sensitivity of this assay was 0.3 pg/ml.

**Measurement of nitric oxide.** Nitric oxide synthesis by cultured macrophages, as reflected by nitric oxide levels in culture supernatants, was assessed by the Griess reaction. Briefly, 100-μl aliquots of supernatants were plated in 96-well microtiter plates. An equal volume of Griess reagent (0.1% naphthalene diamine hydrochloride, 2.5% phosphoric acid, and 1% sulfanilamide dissolved in demineralized water) was added to each well. After 10 min of incubation at room temperature, the optical density was measured in an enzyme-linked immunosorbent assay reader at a wavelength of 540 nm. Samples were tested in triplicate and related to a standard curve included in each experiment.

**Statistical analysis.** All data were analyzed using the Wilcoxon t-test.

## RESULTS

**Inhibition of schizont development by FO supplementation.** In the first experiment, only the FO and CO groups were included. After six weeks of the diet, the rats were injected with 5 × 10⁵ sporozoites intravenously. Fish oil-fed rats developed significantly lower numbers of EEF/cm² of liver section than rats on the CO-supplemented diet (Table 1). These results do not exclude the possibility that the CO-supplemented diet enhanced the development of EEF. Therefore, the experiment was repeated with the addition of a control group, which was fed standard lab chow (LC group). In this experiment, the rats were injected with 4 × 10⁴ sporozoites. The number of EEF/cm² in the control group and in the CO-supplemented group did not differ significantly, but the rats on the FO-supplemented diet had significantly lower EEF densities. The size and appearance of developing schizonts in the three groups was not different, suggesting that the killing effect mediated by FO is only effective in the early stage of development and that schizonts that survive this stage are no longer susceptible.

**Influence of an FO diet on cytokine production capacity of peritoneal macrophages.** The proportion of macrophages in the peritoneal aspirates, as assessed by 𝛽-oxidase staining, was always between 75% and 85%. Cell-associated and secreted IL-1 were measured separately, because IL-1α is hardly secreted in the supernatant. Concentrations of bioactive IL-1 in supernatants of LPS-stimulated macrophages were significantly lower in FO-fed rats than in CO-fed or LC-fed rats (P < 0.05). Concentrations of cell-associated IL-1 were also lower in FO-fed rats, but the difference was not statistically significant (P = 0.06) (Figure 1). Similar
results were obtained for IL-6 levels, which were significantly lower in FO-fed rats (Figure 2).

Influence of an FO diet on LPS-stimulated nitric oxide synthesis by macrophages. It has been demonstrated that nitric oxide strongly inhibits schizont development in hepatic cultures. Its synthesis can be induced in several cell types such as endothelial cells, hepatocytes, and macrophages. To investigate whether increased inducibility of nitric oxide production in macrophages from FO-supplemented rats could explain the reduced schizont development in this group, nitric oxide levels were measured in culture supernatants of peritoneal macrophages with and without LPS stimulation. Coculture with LPS induced a statistically significant increase of about 20%. However, there was no difference in nitric oxide levels between the three dietary groups (Table 2).

**DISCUSSION**

Our results show that an FO-supplemented diet can strongly reduce schizont development upon intravenous administration of *P. berghei* sporozoites in rats. These findings suggest a protective effect of FO on primary malaria infection. We also demonstrated the reduced cytokine production capacity of peritoneal macrophages from FO-supplemented rats. These results corroborate the findings of Billiar and others, who showed reduced IL-1 production by liver macrophages from FO-fed rats. The difference between cell-associated and secreted IL-1 in our study suggests a stronger reductive effect of an FO diet on the production of IL-1β than on IL-1α. This is in agreement with the results of Endres and others and Meydani and others. In their experiments in humans, an FO diet significantly reduced the ex vivo production of TNF IL-1β, and IL-6 by stimulated peripheral blood mononuclear cells. In mice, however, Blik and others found that protection against cerebral malaria was...
associated with increased cytokine production capacity of peritoneal macrophages. This underlines once more that extrapolation of experimental results in an animal model to humans or other species can prove erroneous.

In several in vitro experiments and also in our previous in vivo study, it was demonstrated that exogenous IL-1 and IL-6 both inhibit schizont development. Also, the effect of exogenous IL-1 could be reversed by treatment with anti-IL-6 antibody. Thus, inhibition of schizont development seems to be accomplished by IL-6. In this study, however, an FO-supplemented diet inhibited schizont development, but at the same time reduced cytokine production capacity. This discrepancy urged us to look for an alternative mechanism by which dietary FO supplementation might inhibit schizont development. Recently, Nussler and others demonstrated the inhibitory effect of nitric oxide on schizont development in vitro. We found that nitric oxide concentrations in macrophage culture supernatants from FO-fed rats were similar to those from LC-fed or CO-fed rats. This suggests that the inhibition accomplished by the FO diet is not mediated through cytokines or nitric oxide, but through another yet unknown mechanism, and that this mechanism makes up for the reduced cytokine production.

The effects of dietary polyunsaturated fatty acids on cells concern mainly membrane structure and function, enzyme production, and receptor expression on the cell surface. Expression of several membrane receptors is known to be down-regulated by an FO-supplemented diet. Van Pelt and others identified two membrane proteins in human hepatocytes that are involved in the penetration of sporozoites. Possibly, parallel to other cell surface receptors, sporozoite-specific receptors on the hepatocyte surface may also be down-regulated by an FO-supplemented diet, thus leading to reduced penetration. Kumaratilake and others demonstrated a killing effect of n-3 and n-6 polyunsaturated fatty acids in vitro on P. falciparum and in vivo on P. berghei trophozoites. They showed that this effect was accomplished by oxidation. Levander and Ager also demonstrated the susceptibility of malarial parasites to oxidative stress induced by polyunsaturated fatty acids. Docosahexanoic acid and eicosapentanoic acid are both important components of FO. A direct effect of oxidants on intrahepatic trophozoites has been demonstrated by Pied and others.  Therefore, this mechanism is also a plausible explanation for the effect of an FO diet on schizont development in vivo. Further studies are needed to clarify this phenomenon and to explore its possible clinical use.

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