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Dietary Fish-Oil Supplementation in Experimental Gram-Negative Infection and in Cerebral Malaria in Mice

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Dietary fish-oil supplementation interferes with eicosanoid production and appears to decrease production of interleukin-1 (IL-1) and tumor necrosis factor (TNF). The effect of fish oil was investigated in an intramuscular *Klebsiella pneumoniae* infection in Swiss mice and in cerebral malaria induced by *Plasmodium berghei* in C57B1/6 mice. After a low inoculum of *K. pneumoniae*, 90% of fish oil-fed mice survived; survival in control mice fed equal amounts of corn or palm oil or normal chow was 30%, 40%, and 0, respectively. Cerebral malaria occurred in only 23% of fish oil-fed mice; in the controls, cerebral malaria developed in 61%, 81%, and 78%, respectively. Contrary to what was expected, lipopolysaccharide-induced ex vivo production of IL-1 α and TNF α by peritoneal cells was significantly enhanced in fish oil-fed mice compared with controls. Indomethacin treatment did not alter the outcome in these two infections, thus arguing against reduced prostaglandin synthesis as an explanation for the increase in resistance to infection.

In a variety of experimental and clinical situations, dietary fish-oil supplementation has been shown to inhibit inflammation. In mice, development of lupus nephritis and induction of amyloidosis were significantly inhibited; in guinea pigs, survival after endotoxin administration was enhanced [1-3]. Moreover, the febrile response to interleukin-1 (IL-1) was attenuated by a fish oil-enriched diet in guinea pigs [4]. In clinical studies, rheumatoid arthritis was alleviated and psoriasis improved after 6-8 weeks of treatment with a fish-oil concentrate [5, 6]. In human volunteers, there was a slightly reduced febrile response to endotoxin [7]. The reduced inflammation induced by fish oil has been attributed mainly to alterations in eicosanoid metabolism. Eicosapentaenoic acid is regarded as the active fatty acid in fish oil because of its structural similarity to arachidonic acid, the usual substrate for the enzymes cyclooxygenase and lipoxygenase. After prolonged ingestion of fish oil, the production of prostaglandin E₂ (PGE₂) and leukotriene (LT) B₄ is markedly reduced, while the biologically less active PGE₃ and LTB₅ are formed [8, 9].

More recently, alterations have been described in the production of the proinflammatory cytokines IL-1 and tumor necrosis factor- α (TNF α) after a fish oil-supplemented diet. In rats, production of these cytokines by Kupffer cells was significantly decreased after 6 weeks of a fish oil-enriched

diet [10]. In humans, 6 weeks of dietary fish-oil supplementation resulted in a decreased ex vivo production of IL-1 α and - β and TNF α by stimulated peripheral blood mononuclear cells, the effect being even more pronounced 10 weeks after cessation of the diet [11]. In contrast, IL-1 and TNF synthesis by mouse peritoneal macrophages was reported to be enhanced after 16 days of a fish oil-enriched diet when compared with controls receiving corn oil [12].

In many forms of severe infection, important roles for IL-1 and TNF have been postulated. Circulating levels of IL-1 β and TNF were elevated in septic patients and after endotoxin infusion in human volunteers [13]. Elevated concentrations of TNF have been noted in the serum of malaria patients and in children with *Plasmodium falciparum* malaria, serum TNF concentrations correlated with disease severity [14]. Antibodies to TNF have prevented death in experimental gram-negative infection and in experimental cerebral malaria [15, 16]. Because of the role of these cytokines in infection and the modulation of their production by fish oil, we investigated the effects of a fish oil-enriched diet on resistance to infection in two experimental models, lethal gram-negative infection and cerebral malaria in mice.

Methods

Gram-negative bacterial infection. Female Swiss mice of ~25 g were obtained from a local colony and housed under specific pathogen-free conditions. *Klebsiella pneumoniae* (ATCC 43816), a strain that produces a lethal infection in normal mice, was inoculated in the left thigh muscle of nonneutropenic mice as described elsewhere [17]. Inocula ranged from 0.5 \times 10³ to 0.5 \times 10⁶ cfu. Survival was scored up to 5 days after infection.

Cerebral malaria. For the malaria experiments, an inbred

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susceptible mouse strain had to be used. Female C57B1/6J mice of ~20 g were obtained from a local colony and housed under specific pathogen-free conditions. *Plasmodium berghei* K173 was maintained by weekly transfer of parasitized erythrocytes from infected to naive mice. Mice were infected with 1000 parasitized erythrocytes intraperitoneally. In this model, death occurs in the second week after infection and is associated with a collapse of thermoregulation [18]. Histologically, the brains of these animals show loss of endothelial cell wall integrity, perivascular edema, hemorrhages, and sequestration of leukocytes in the cerebral vessels [19]. On days 7, 9, and 11 after infection, thin blood films were made from tail blood and stained with May-Grünwald and Giemsa solutions to score parasitemia. After death, light and electron microscopy of brain tissue using routine histologic procedures was done to detect cerebral hemorrhages.

Diets. In the first series of experiments, mice were fed standard lab chow (Hope Farms, Woerden, Netherlands) with daily supplementation of 0.2 ml of a fish-oil concentrate (Mepatrin, gift of Sanofi, Maassluis, Netherlands, and Epax, gift of Jahres Fabrikker, Sandefjord, Norway; both preparations contained 34% eicosapentaenoic acid and docosahexanoic acid) administered by gastric instillation. Control animals received 0.2 ml of palm oil (Kriskol, gift of Loders-Croklaan, Wormerveer, Netherlands) or corn oil (Mazola; Knorr, CPC, Utrecht, Netherlands) by the same route. Oil supplementation represented ~10% of total caloric intake.

In subsequent experiments, the animals were fed a fat-free standard reference diet as a dry powder (Hope Farms), supplemented (wt/wt) with 14% fish oil and 1% corn oil (fish-oil group), 15% corn oil (corn-oil group), 15% palm oil (palm-oil group), or standard lab chow without oil supplementation. In these experiments, oil supplements represented ~28% of total caloric intake. Twice a week, the food containing the supplements was freshly prepared and stored at 4°C. In the malaria experiments, *p*-aminobenzoic acid (PABA) was added to the drinking water to overcome the PABA-deficiency of the standard reference diet, which otherwise would have interfered with parasite proliferation. Diets were started 6 weeks before infectious challenge and continued thereafter.

Indomethacin. In separate experiments, the effects of inhibition of prostaglandin production were evaluated. In these experiments, mice were fed normal lab chow. In the *K. pneumoniae* infection, indomethacin at 1 mg/kg, dispersed in 0.2 ml of carboxy-methyl cellulose 1% (CMC), was given by gastric instillation once a day starting 6 h before infection. In the malaria experiment, the same dosage of the drug was administered daily from infection until death. Control animals received the same volume of CMC only.

IL-1 and TNF. After 6 weeks on the diets, subsets of mice were killed and peritoneal cells were harvested by rinsing, using cold PBS with 0.38% sodium citrate as described elsewhere [20]. Cells were then centrifuged and resuspended in RPMI 1640 (Dutch modification; Flow Laboratories, Irvine, UK), containing 1% pyruvate, 1% glutamate, and 1% gentamicin (Essex, Amstelveen, Netherlands). After counting, the concentration was adjusted to 2×10^6 cells/ml, and volumes of 100 μ l of cell suspensions were layered onto round-bottom microtiter plates

(Greiner, Nürtingen, Germany) to which either 100 μ l of medium or medium containing *Escherichia coli* (serotype O55:B5) lipopolysaccharide (LPS; Sigma, St. Louis) at a final concentration of 100 ng/ml was added. After 24 h of culture at 37°C in a water-saturated atmosphere with 5% CO₂, the culture supernatants were harvested and frozen at -70°C until assay; 200 μ l of medium was added to the adherent cells and these cells were frozen at -70°C. Three freeze-thaw cycles were done before assay of cell-associated cytokines.

Mouse IL-1 α and -1 β were determined by specific RIAs [21]. Murine recombinant IL-1 α (rIL-1 α ; gift of P. Lomedico, Hoffmann-Laroche, Nutley, NJ) and murine rIL-1 β (gift of A. Shaw, Glaxo, Geneva) were labeled with ¹²⁵I by a modification of the chloramine-T method and purified by chromatography over Sephadex G-50. All antibodies were prepared at our institute. The assay buffer consisted of 0.01 M sodium phosphate in 0.9% saline (pH 7.4) with 0.25% bovine serum albumin (BSA; Sigma) and 0.25% sodium azide. Reference standards from 10⁴ to 40 pg/ml were prepared in RPMI 1640 by serial twofold dilutions of mouse rIL-1 α and -1 β . Samples of 100 μ l were assayed in duplicate or in triplicate. Samples from LPS-stimulated cells were assayed undiluted as well as in a 1:4 dilution in RPMI. An aliquot (100 μ l) of specific polyclonal antibody (rabbit anti-mouse IL-1 α or -1 β , respectively), diluted appropriately in assay buffer, was added to the reference standards and to the samples. After 24 h of incubation at room temperature, 10⁴ cpm of radio-labeled IL-1 α or -1 β in 100 μ l of assay buffer was added. After a further 2 days of incubation at room temperature, the first antibody was precipitated by adding 0.75 ml of a solution containing 1.6% goat anti-rabbit IgG and 9% polyethylene glycol (16–20 kDa; Sigma). After incubation at room temperature for 1 h, samples were centrifuged at 1500 g for 15 min, and the radioactivity pelleted in the assay tubes was counted in a γ counter. Nonspecific binding (NSB) in the absence of first antibody was subtracted and the result expressed as a percentage of the zero standard (B0) minus NSB. Standard curves were plotted as percentage of B0 against cytokine concentration, and cytokine concentrations were read from the linear part of the curve. Sensitivity of the assay was 80 pg/ml for IL-1 α and 320 pg/ml for IL-1 β . When the original sample was diluted, the value is expressed as that of the original concentration.

Mouse TNF α was determined by ELISA as described elsewhere [22]. In short, 96-well immunoassay plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with TN3, a hamster monoclonal antibody specific for murine TNF and lymphotoxin (kindly provided by K. C. F. Sheehan and R. D. Schreiber, Celltech, Slough, UK) [23]. Plates were blocked with 1% BSA. After four washings with wash-buffer, test samples were added to the plate for 1 h at room temperature. A standard titration curve was obtained by serial dilution of a known sample of recombinant murine TNF (Genzyme, Cambridge, MA) in medium identical to that of the test sample. Plates were then washed four times with wash-buffer and incubated with rabbit anti-mouse TNF immune serum (Genzyme) and peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA). After adding the substrate (*o*-phenylenediamine; Sigma) for 10 min, the color reaction was stopped with 1.0 M H₂SO₄ and photometry (492 nm) was done. The ELISA has a lower detection limit of 150 pg/ml.

Organ counts of bacteria. Forty-eight hours after the injection of *K. pneumoniae*, mice fed fish oil and control mice were anesthetized with ether and bled from the retrobulbar vessels by eye extraction. Blood for cultures was taken and livers and spleens were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. To bring the bacterial counts after culture into the optimal range for reading, samples of liver and spleen were diluted in sterile saline. The suspensions were plated on sheep blood agar, and after overnight incubation the colony-forming units were counted.

Statistical analysis. Survival curves were analysed using the Kaplan-Meier log rank test [24]. Other differences between groups were analysed using the Kruskal-Wallis test with χ^2 approximation. Differences between groups were tested for significance only when overall $P < .05$ (regarded as significant).

Results

***K. pneumoniae* infection.** After 6 weeks of oil supplementation by gastric instillation, no differences in survival between fish, corn, or palm oil-fed mice were seen. In this experiment, the *K. pneumoniae* inoculum was 10^5 cfu and the median survival was 32 h. In subsequent experiments, oil supplementation was increased to 15% (wt/wt). Using lower inocula of this organism, fish oil-fed mice survived longer than control groups (figure 1A). The effect was most prominent with the lowest bacterial inoculum used (figure 1B, $P < .05$). No differences in the numbers of *K. pneumoniae* colony-forming units in blood, liver, and spleen were found between the mice fed fish oil or normal lab chow. Indomethacin (1 mg/kg), given daily from infection until death, did not influence survival after *K. pneumoniae* infection (data not shown).

Cerebral malaria. In the second week of the infection, 6 (31.5%) of 19 mice receiving the fish oil supplementation by gastric instillation developed the cerebral malaria syndrome compared with 18 (95.7%; $P < .001$) of 19 mice in the palm oil-fed group and 4 of 5 mice in the control group that did not receive dietary intervention. Cerebral malaria was suspected in animals that died in the second week after infection and was confirmed histologically. Parasitemia in the fish oil-fed mice was significantly lower than in the other diet groups (fish oil vs. no oil, $P < .01$ on days 7 and 9; fish oil vs. palm oil, $P < .05$ on day 9). On days 7 and 9, parasitemia was significantly lower in the fish-oil group compared with the other groups.

In mice fed a fat-free reference diet supplemented with 15% fat (wt/wt), cerebral malaria occurred in 7 (23.3%) of 30 animals in the fish-oil group, in 20 (60.6%) of 33 in the corn-oil group (fish oil vs. corn oil, $P < .02$), and in 25 (80.6%) of 32 in the palm-oil group (fish oil vs. palm oil, $P < .001$), while 18 (78.3%) of 23 mice in the group fed normal lab chow died of cerebral malaria (fish oil vs. normal lab chow, $P < .001$). Although at days 9 and 11 parasitemia was lower in

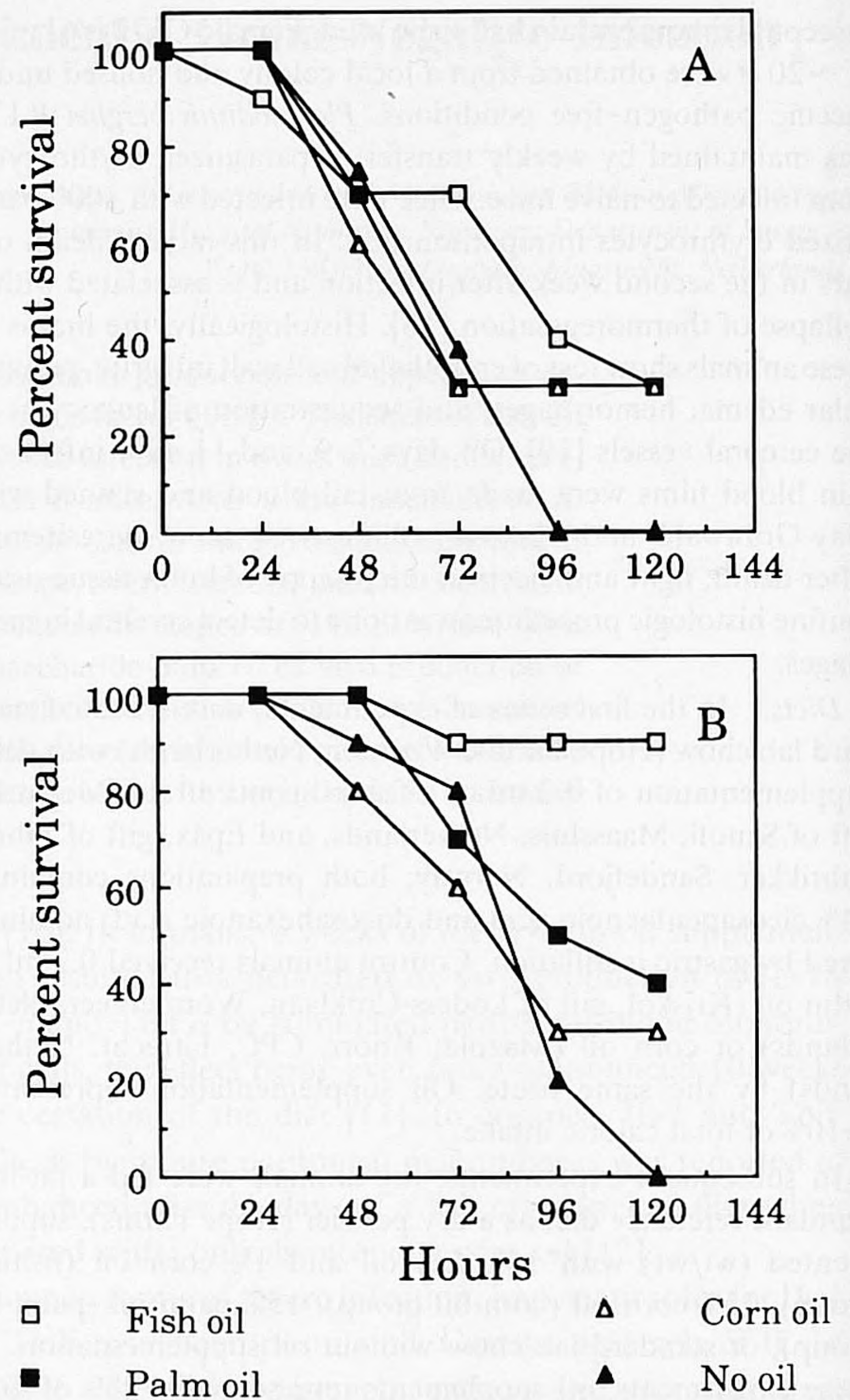


Figure 1. Survival of Swiss mice after intramuscular administration of (A) 0.5×10^4 and (B) 0.5×10^3 cfu of *Klebsiella pneumoniae*. Oil supplements constituted 15% of the diet (wt/wt). Results of repeated experiments; each group consists of at least 10 mice. In B, difference in survival between fish-oil fed mice and other diet groups is significant ($P < .05$).

the fish-oil group than in the other groups, the differences were not significant (figure 2).

Indomethacin. Of 23 mice treated with indomethacin, 15 (62.2%) developed cerebral malaria compared with 11 (57.9%) of 19 animals in the control group. Parasitemias between the two groups did not differ significantly (data not shown).

IL-1 α and -1 β and TNF. The effects of the different diets on the concentrations of cytokines associated with resident peritoneal cells cultured for 24 h and the concentrations in the supernatants are depicted in figure 3. Concentrations of LPS-stimulated, cell-associated IL-1 α were significantly higher in animals fed fish oil than in those fed corn oil ($P < .02$) or palm oil ($P < .01$). Concentrations of secreted IL-1 α were low and did not differ between groups. On average, IL-1 β concentrations were higher than those of IL-1 α ; cell-

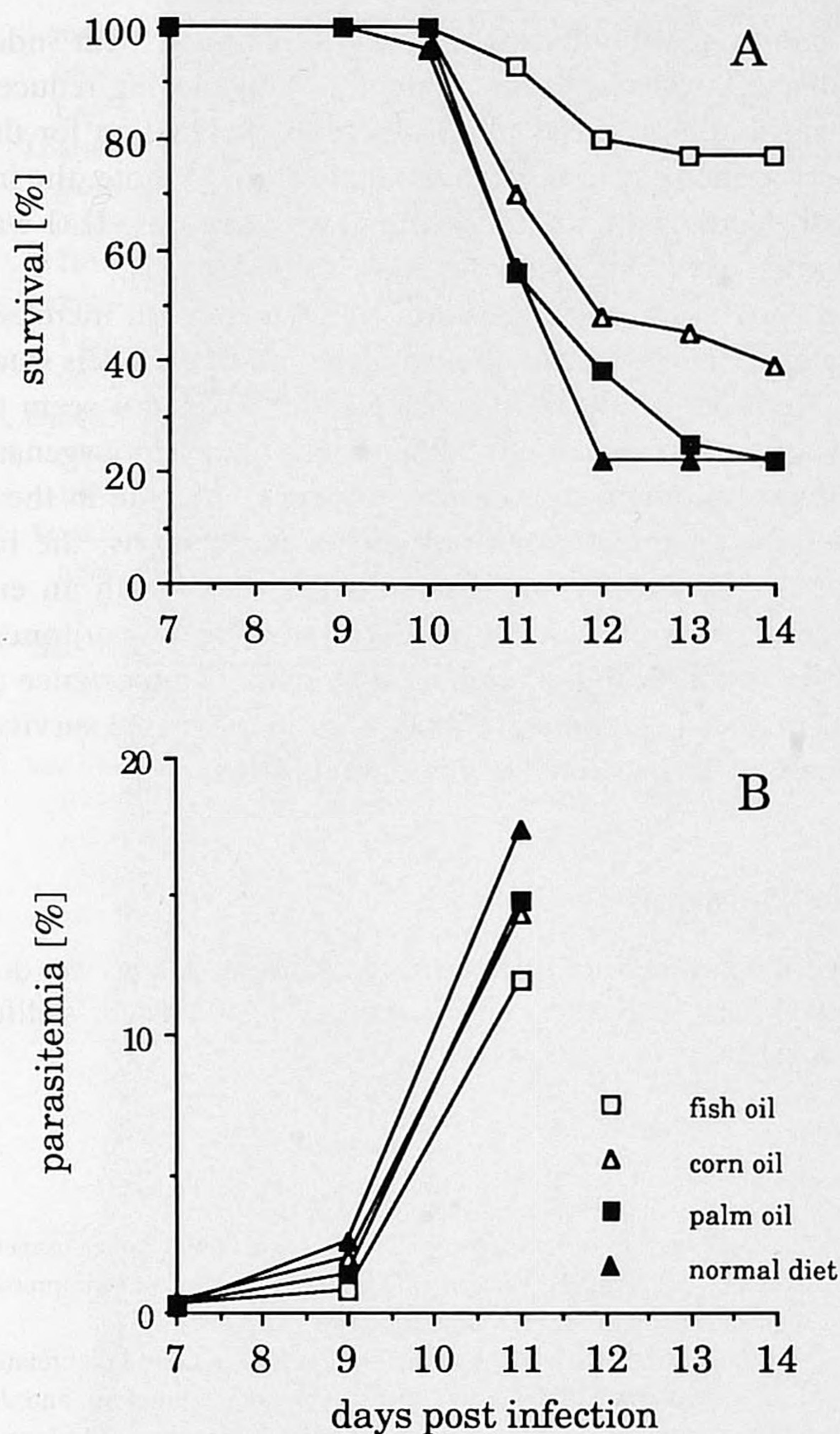


Figure 2. A, Survival of C57Bl/6 mice in the second week after infection with 10^3 erythrocytes parasitized with *Plasmodium berghei*. Diets were supplemented with 15% fish oil, corn oil, palm oil (wt/wt), or normal lab chow (fish oil vs. corn oil, $P < .02$; fish oil vs. palm oil, $P < .001$; fish oil vs. normal lab chow, $P < .001$). B, Parasitemia expressed as percentage of erythrocytes parasitized; in this experiment, differences were not statistically significant.

associated IL-1 β was the highest in palm oil-fed mice and secreted IL-1 β the highest in the fish-oil group. These differences were not statistically significant. In the three experimental groups, concentrations of cell-associated TNF were low and mostly below the detection limit (data not shown). In contrast, concentrations of secreted TNF, both unstimulated and stimulated, were significantly higher in the fish-oil group compared with the palm-oil and corn-oil groups ($P < .02$ and $< .01$, respectively).

Discussion

We have shown that raising the content of n-3 polyunsaturated fatty acids in the diet during a period of 6 weeks in-

creased resistance to infection in two experimental models in mice, lethal *K. pneumoniae* infection and cerebral malaria. The results indicate that the immunomodulatory and possibly immunosuppressive effects of dietary fish-oil supplementation did not lead to decreased resistance to infection. These results are in agreement with the finding of increased survival of endotoxin shock following dietary fish-oil supplementation [3].

Rubin et al. [25] did not find differences in the susceptibility of (NZBxNZW)F1 mice to *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Candida albicans*, or murine cytomegalovirus after 4 weeks of a fish oil-enriched diet. In their study, fish-oil supplementation may have had no effect because of the shorter duration of supplementation. In at least one other study, the antiinflammatory effect of fish oil did not become apparent until fish-oil feeding had been sustained for 6 weeks [4].

In the *K. pneumoniae* infection, protection in the fish oil-fed group was most prominent when the bacterial inoculum

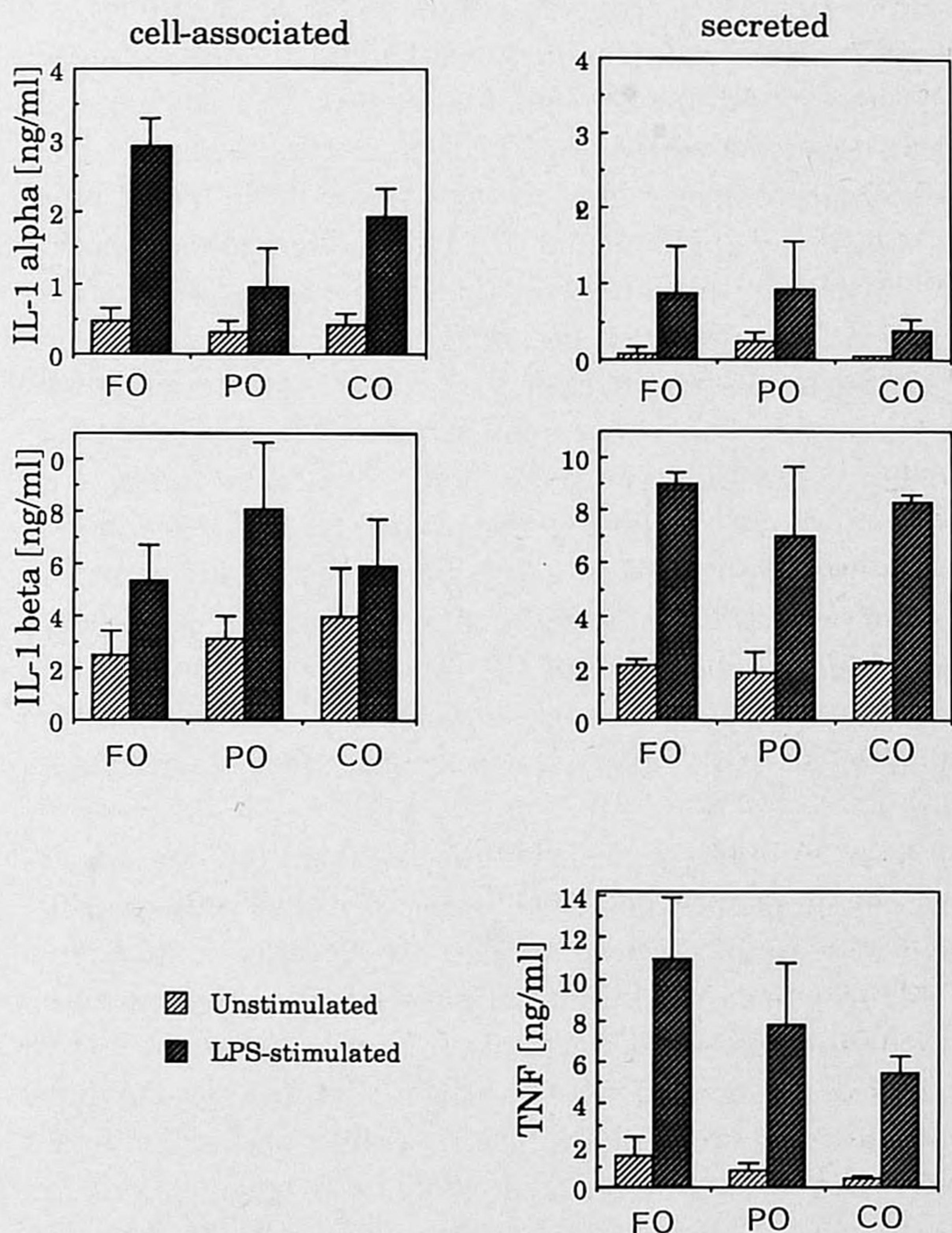


Figure 3. Concentrations of interleukin (IL)-1 α , IL-1 β , and tumor necrosis factor (TNF) after culture of peritoneal cells for 24 h in presence or absence of lipopolysaccharide (LPS, 100 ng/ml). Values for LPS-stimulated, cell-associated IL-1 α were significantly higher for fish oil (FO) compared with palm oil (PO; $P < .01$) or corn oil (CO; $P < .02$). Values for secreted TNF were significantly higher in FO group compared with PO group ($P < .02$) or CO group ($P < .01$). $n = 5$ for each group.

was low, resulting in a longer median survival in all experimental groups. Whatever mechanism is involved in the increased resistance to this bacterial infection after fish-oil feeding, it is clear that it does not become apparent when the course of infection is rapidly fatal.

Dietary fish-oil supplementation has been shown to reduce the production of eicosanoids like PGE₂. Because increased survival to experimental endotoxin shock after treatment with a cyclooxygenase inhibitor has been reported [26], we investigated whether a cyclooxygenase inhibitor such as indomethacin would influence survival in our models. The absence of an effect of indomethacin on survival in these infections argues against reduced prostaglandin synthesis as an explanation for the increased resistance to infection.

In contrast to what was expected, the increase in survival in the *K. pneumoniae* infection and the *P. berghei* infection was not associated with decreased production of IL-1 and TNF by peritoneal cells. In fact, the cell-associated IL-1 α and TNF production capacity of these cells was enhanced in the fish oil-fed mice. These findings are in disagreement with the reports of reduced IL-1 and TNF production by rat Kupffer cells and human peripheral blood mononuclear cells after dietary fish-oil supplementation [10, 11, 27]. One study reported increased IL-1 and TNF production by mouse peritoneal macrophages, but cells were studied after only 16 days of a fish oil-supplemented diet [12]. Differences in species, cell types, and durations of diets should be considered to explain these divergent findings.

In the models of infection studied here, protection by low dosages of exogenously administered IL-1 has been well documented [28–30]. The protection provided by fish-oil supplementation is similar to the protection by IL-1 administered exogenously 24 h before gram-negative infection and early in the course of experimental malaria. An exaggerated endogenous production of IL-1 and TNF early in infection induced by dietary fish-oil supplementation may have the same effect as the exogenous administration of these cytokines.

Apart from interference with cytokine production, dietary fish-oil supplementation has been associated with attenuation of some of the responses to exogenously administered IL-1. In guinea pigs, the febrile response to rIL-1 injection was attenuated after 6 weeks of a fish oil-enriched diet [4]. In rats, feeding fish oil for 6 weeks attenuated the catabolic response to a combined infusion of rIL-1 and recombinant TNF, as reflected by reduced whole-body leucine oxidation and increased net hepatic protein anabolism [31]. Also, anorexia induced by IL-1 was reduced in rats following a fish oil-enriched diet [32].

Because many of these effects have also been observed after pretreatment with a cyclooxygenase inhibitor before injection of IL-1 [33, 34], modulation of these IL-1 effects by fish-oil supplementation could be due to the decreased prostaglandin production after the fish oil-supplemented diet.

As mentioned before, in our study, treatment with indomethacin did not influence outcome, thus making reduced prostaglandin synthesis not a plausible explanation for the observed increase in resistance to infection. Of note, the increase in survival after pretreatment with low-dose IL-1 was not influenced by a cyclooxygenase inhibitor [17].

In conclusion, dietary fish-oil supplementation increases resistance to infection in the two experimental models studied. Reduced synthesis of prostaglandins does not seem to play a role here because treatment with the cyclooxygenase inhibitor indomethacin did not influence outcome in these experimental infections. Contrary to expectations, the increased resistance to infection was associated with an enhanced *ex vivo* production of IL-1 and TNF by peritoneal cells in the fish oil-fed animals. The relative importance of this enhanced cytokine production in the increased survival in these infections remains to be established.

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