Dietary n-3 Fatty Acids Increase Spleen Size and Postendotoxin Circulating TNF in Mice; Role of Macrophages, Macrophage Precursors, and Colony-Stimulating Factor-1

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In experimental studies in mice, dietary supplementation with n-3 fatty acids (FA) alleviates inflammation and increases resistance to infection. Nevertheless, TNF production capacity was found to be increased in n-3 FA-fed mice. We previously found increased relative spleen weights in n-3 FA-fed mice. In this study, the nature of this increased spleen size was further investigated. Spleen cellularity was increased significantly in mice fed n-3 FA (fish oil 15% w/w), compared with controls fed corn oil (15%) or normal lab chow (p < 0.05). Experiments with T cell-deficient nude mice and experiments using macrophage depletion through liposomal dichloromethylene-bisphosphonate revealed that the increase in spleen cellularity is T cell independent and largely due to macrophage accumulation in the spleen. Accumulation of marginal zone and red pulp macrophages was histologically and immunohistochemically confirmed. n-3 FA induced peripheral blood monocytes and an aspecific increase in bone marrow cellularity. Postendotoxin circulating TNF concentrations were increased significantly in n-3 FA-fed mice compared with controls. Splenectomy did not abolish this increase in circulating TNF. However, after macrophage depletion through liposomal dichloromethylene-bisphosphonate, circulating TNF was not detectable after endotoxin challenge. Circulating concentrations of CSF-1 did not differ between the various experimental groups. It is suggested that the cellular changes observed relate to increased constitutive production of TNF.


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Abbreviations used in this paper: FA, fatty acid; CI2 MDP, dichloromethylene-bisphosphonate; CO, corn oil; FO, fish oil.

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The mice were anesthetized with ether, and blood was taken from the retrobulbar vessels after eye extraction. The animals were killed by neck dislocation, and spleens were removed and weighed. Total body weight was determined immediately before bleeding, and relative spleen weight was calculated afterward. A suspension of spleen cells was made over a nylon filter, and cells were counted with a Coulter counter (Coulter Corp., Hialeah, FL). The effect of dietary n-3 FA on spleen weight was determined in normal Swiss mice, normal BALB/c mice, and T cell-deficient nude BALB/c mice (nu/nu).

Spleen cells were phenotypically characterized with flow cytometry using a set of mAbs specific for mouse cells: 59-AD 2,2 detecting Thy-1 (T lymphocytes), RA3 6B2 detecting B220 (B lymphocytes), R129.19 detecting MT4 (CD4-positive T lymphocytes), 53-6.72 detecting Ly-2 (CD8-positive T lymphocytes), KT3 (CD3-positive T lymphocytes), and F4/80 (macrophages). Details concerning these Abs can be found in Leenen et al. (24).

After immunohistochemical staining of spleen sections with RA3 6B2 (anti-B220), a mAb specific for mouse B cells, the relative surface area of white pulp was quantitated using a Leitz Diaplan light microscope (Leitz, Wetzlar, Germany) and a Videoplan image-processing system (Kontron, Munich, Germany).

To analyze the role of mature phagocytic macrophages in the increase in spleen size, mice were fed FO for 0, 2, or 4 wk. Subsequently the animals were injected i.v. in the tail vein with 1 mg of liposomal dicholoromethylene-bisphosphonate (Cl1, MDP or elodronate), 24 h before LPS challenge, to deplete macrophages (25). Control animals receiving the same diet were injected simultaneously with saline. Liposomal Cl1, MDP was prepared as described (25). Spleen histology was studied in treated and untreated animals to verify macrophage depletion.

Peripheral blood cells
Peripheral blood cell count was performed by separately determining hematocrit and the total white blood cell count (using Coulter counter). Differential white blood cell count was performed by quantifying the light scatter profile of nucleated cells.

Bone marrow analysis
Bone marrow was obtained from both femora of each mouse by flushing the femur shafts with 3 ml of culture medium (RPMI, Dutch modification; Flow Laboratories, Irvine, Scotland). Nucleated cells were counted with a Coulter counter. Soft agar cultures of bone marrow cells were stimulated with macrophage CSF for 2 wk, and the number of macrophage precursors in the original preparation was quantitated by counting the number of macrophage colonies and clusters (<50 cells); macrophage colony-forming cells and macrophage cluster-forming cells are given per 10^6 nucleated bone marrow cells (26).

Department of Immunology (Erasmus University, Rotterdam, The Netherlands), as described (27). The following mAbs were used: ER-MP12 and ER-MP20, identifying subpopulations of macrophage precursors; ER-MP58, identifying myeloid cells; and ER-MP21, identifying transferrin receptor-expressing cells (28).

Postendotoxin circulating TNF
At a time period of 1.5 h after i.p. administration of 10 μg of LPS (Escherichia coli, serotype O55:B5; Sigma Chemical Co., St. Louis, MO), mice were anesthetized with ether, and blood was taken from the retrobulbar vessels after eye extraction. Blood samples (approximately 1 ml) were mixed with 100 μl of EDTA solution (21.4 mg/ml of EDTA in H2O) and kept on ice until centrifugation at 1500 × g for 5 min. Resultant plasma was isolated and stored at −20°C until assay.

TNF-α was measured by ELISA using TN3, a hamster mAb specific for murine TNF-α, and lymphotoxin, as described (29, 30).

In separate experiments, the effect of splenectomy on postendotoxin circulating TNF was studied. Splenectomy was performed after ether anesthesia in 4-wk-old mice, 2 wk before the animals were started on the experimental diets.

Colonyl-stimulating factor-1
CSF-1 was determined in unstimulated serum samples by specific RIA, as described in detail elsewhere (31). This assay is based on the competition by CSF-1 for the interaction between 125I-labeled, purified mouse L cell CSF-1 glycoprotein and a rabbit polyclonal Ab to purified L cell CSF-1 (32), and is more sensitive than the conventional CSF-1 bioassay based on bone marrow colony formation. Assays were conducted in duplicate on 20-μl samples. The concentration of CSF-1 in U/ml (1 U = 12 pg) was determined with reference to a standard curve prepared using a stable, partially purified L cell CSF-1 preparation.

Statistical analysis
Differences between groups were analyzed using the Kruskal-Wallis non-parametric ANOVA test, corrected for ties. Results were considered statistically significant at p < 0.05.

Results
Effects of FO supplementation on spleen cellularity
The effects of dietary n-3 FA on spleen, peripheral blood, and bone marrow cells are summarized in Table I. The following experiments were done to elucidate the mechanisms of the increased spleen weight in FO-fed mice. After 4 wk of dietary supplementation with FO or CO, experimental Swiss mice were compared with control animals on normal lab chow. FO-fed mice had significantly increased relative spleen weights (p = 0.01) and spleen

<p>| Table I. Effect of dietary n-3 FA on spleen, peripheral blood, and bone marrow cells |
|----------------------------------|-------------------------------|-------------------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Fish oil</th>
<th>Corn oil</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative spleen weight (mg/g)</td>
<td>5.9 ± 0.5</td>
<td>4.8 ± 0.3</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Spleen cellularity (× 10^6)</td>
<td>2.7 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>B 220 positive spleen cells (%)</td>
<td>41 ± 4</td>
<td>36 ± 2</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White blood cell count (× 10^9/ml)</td>
<td>43 ± 2.6</td>
<td>42 ± 3.2</td>
<td>43 ± 1.6</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>12.2 ± 4.0</td>
<td>9.1 ± 1.9</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>55 ± 8</td>
<td>69 ± 4</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>34 ± 8</td>
<td>25 ± 4</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellularity (× 10^6)</td>
<td>24 ± 5</td>
<td>21 ± 7</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>M-CFC (No. macrophage precursors/10^6 NBMC)</td>
<td>58 ± 15</td>
<td>51 ± 17</td>
<td>77 ± 20</td>
</tr>
<tr>
<td>M-clustFC (No. macrophage precursors/10^6 NBMC)</td>
<td>94 ± 22</td>
<td>80 ± 16</td>
<td>98 ± 27</td>
</tr>
<tr>
<td>ER-MP 21 high (erythroid cells) (%)</td>
<td>22 ± 5</td>
<td>25 ± 3</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>ER-MP 58 high (myeloid cells) (%)</td>
<td>50 ± 7</td>
<td>50 ± 2</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>ER-MP 12 high/20 neg (committed progenitors) (%)</td>
<td>1.2 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>ER-MP 12 pos/20 pos (immature myeloid precursors) (%)</td>
<td>5.4 ± 1.2</td>
<td>5.6 ± 0.5</td>
<td>5.0 ± 1.2</td>
</tr>
<tr>
<td>ER-MP 12 neg/20 high (monocytes) (%)</td>
<td>5.7 ± 0.9</td>
<td>6.2 ± 0.8</td>
<td>5.7 ± 1.6</td>
</tr>
</tbody>
</table>
cellularity \((p = 0.02)\). In separate experiments, time-effect relationships in these responses were investigated. Mice were studied simultaneously after 0, 2, 4, and 6 wk of dietary FO supplementation. Increases in spleen weight were most prominent after 2 wk, and appeared somewhat blunted after 6 wk (Fig. 1A). Similar results were obtained in normal BALB/c mice. To investigate whether the increase in spleen size was accounted for or mediated by T cells, T cell-deficient female BALB/c mice were fed FO for 0, 2, or 4 wk. Relative spleen weight was increased significantly from 2 wk on, indicating that the increase in spleen weight was independent of the presence of T cells (results not shown).

Phenotypic characterization of spleen cells using a set of mAbs revealed no percentual differences in T lymphocytes or macrophages between the various dietary groups. However, FO-fed mice had a small but significant increase in B220-positive B lymphocytes (Table I).

Pretreatment with liposomal C12MDP 24 h before endotoxin challenge did not significantly affect spleen weight. However, in mice that had been on FO diet for 2 or 4 wk, liposomal C12MDP resulted in a decrease in spleen weight (Fig. 2A). In these FO-fed mice, treatment with liposomal C12MDP resulted in spleen weights that were not significantly different from mice that had been on normal diet only. Depletion of marginal zone and red pulp macrophages was histologically confirmed, but accurate quantitation of F4/80-positive macrophages was not possible in cell suspensions, in which their numbers were equal in control and FO-fed mice. However, for other reasons (see below), the results indicate that the increase in spleen weight in FO-fed mice is mainly attributable to an increase in the splenic macrophage population that is depleted by pretreatment with liposomal C12MDP.

To a lesser extent, B lymphocytes seem to contribute to the increased spleen cellularity in FO-fed mice. Quantification of the relative surface area of red and white pulp in spleen sections indicated a decrease in T and B cell-containing white pulp (Fig. 3).

To investigate a possible relation between circulating blood cells and changes in spleen cellularity, peripheral blood cells were quantitated. Hematocrit did not differ between the various treatment
groups. White blood cell count was higher in FO-fed mice, but this difference did not reach statistical significance. Analysis of the light-scatter profile of peripheral blood cells showed a significant increase in percentage of monocytes in FO-fed mice ($p = 0.02$, $n = 6$).

The effect of FO supplementation on bone marrow cells

Possible relations between changes in spleen and peripheral blood cellularity and the cellular composition of bone marrow were investigated. Bone marrow cellularity was increased significantly in FO-fed mice compared with mice fed CO or normal lab chow. This increase in bone marrow cellularity was equally distributed over the various hemopoietic lineages, since differential flow-cytometric analysis of bone marrow cells, using ER-MP12 and ER-MP20 mAbs, showed no significant differences between the various experimental groups (Table I). Moreover, soft agar cultures of bone marrow cells did not reveal a relative increase in macrophage precursors, measured as macrophage colony-forming cells or macrophage cluster-forming cells.

The effect of FO supplementation on postendotoxin TNF

To investigate the suggested increase in mononuclear phagocyte functionality, we measured circulating TNF concentrations after endotoxin challenge. Postendotoxin concentrations of circulating TNF were significantly higher in FO-fed mice than in control mice. Postendotoxin TNF was $10.4 \pm 6.6$ ng/ml in FO-fed mice, $2.2 \pm 1.4$ in CO-fed mice, and $2.7 \pm 1.3$ in mice fed normal diet (mean $\pm$ SD, $p < 0.05$). In separate experiments, time-effect relationships in TNF production and FO diet were investigated. Similar to the increase in spleen weight, increases in postendotoxin circulating TNF were most prominent after 2 wk of FO diet, and appeared somewhat blunted after 6 wk (Fig. 1B). Postendotoxin TNF in T cell-deficient nude BALB/c mice was increased significantly from 2 wk on, indicating that the increase in postendotoxin circulating TNF was independent of the presence of T cells (results not shown).

Splenectomy, performed 2 wk before starting on the FO-supplemented diet, did not influence the increase in postendotoxin circulating TNF concentrations after 2 and 4 wk of diet: at both time points, postendotoxin circulating TNF concentrations were increased significantly compared with baseline (results not shown). At baseline and at 2 and 4 wk of dietary FO supplementation, postendotoxin circulating TNF was not detectable in liposomal C12MDP-treated mice (Fig. 2B). These observations suggest that splenic macrophages, despite their accumulation after dietary FO supplementation, are not the most important contributors to the production of circulating TNF. On the other hand, the macrophage population that is depleted by liposomal C12MDP and that does not reside in the spleen appears to be the most important producer of postendotoxin circulating TNF.

The effect of FO supplementation on circulating concentrations of CSF-1

The mononuclear phagocyte growth factor, CSF-1, is required for the development of the majority of mouse macrophages (33). To accurately measure circulating concentrations of CSF-1, serum samples were subjected to a mouse CSF-1-specific RIA. In biological samples, this RIA detects only biologically active CSF-1 (32, 34), including both glycoprotein and proteoglycan forms (35). Circulating concentrations of immunoreactive CSF-1 did not differ among the various diet groups: $702 \pm 89$ U/ml in FO-fed mice, $753 \pm 96$ in CO-fed mice, and $802 \pm 158$ in mice fed normal diet (mean $\pm$ SD, $p = 0.40$).

Discussion

The present study shows that dietary n-3 FA supplementation has a significant effect on the generation and distribution of mononuclear phagocytes in mice: n-3 FA induce a generalized increase in bone marrow cellularity, peripheral blood mononcytosis, and accumulation of macrophages in the spleen, leading to an increase in spleen size. What could be the mechanism of these n-3 FA-induced changes?

The specific increase in bone marrow cellularity without preferential stimulation of monocytopoiesis is in accordance with our observation on CSF-1: this specific monocyte growth factor was not increased in the n-3 FA-fed mice. The generalized increase in bone marrow cellularity suggests that earlier, broad spectrum hemopoietic growth factors such as IL-3 and granulocyte-macrophage CSF are involved. The production of these factors is largely regulated by proinflammatory cytokines including IL-1 (36, 37). We have shown previously that IL-1$\alpha$ production capacity is increased in mice after dietary n-3 FA (15). Increased constitutive production of this cytokine may be involved in the increased bone marrow cellularity observed in the present study. Moreover, Pelus and others have shown that PGE$_2$ has an important modulatory effect on hemopoiesis in mice: exogenous administration of PGE$_2$ reduced nucleated bone marrow and splenic cellularity, while blockade of PGE$_2$ biosynthesis increased bone marrow and splenic cellularity, especially in the presence of IL-1$\alpha$ (38–42). These observations may partly explain the results obtained in the present study, since dietary supplementation with n-3 FA in mice basically leads to increased IL-1$\alpha$ and a decreased PGE$_2$ production capacity.

Peripheral blood mononcytosis induced by dietary n-3 FA has not been described before, but may be induced through a feedback mechanism following the accumulation of macrophages in the red pulp of the spleen. It is apparently not due to increased concentrations of CSF-1. This is in accordance with the observation that the number of red pulp macrophages is reduced only slightly in CSF-1-deficient op/op mice (43). Other factors, including IL-3, granulocyte-macrophage CSF, and the proinflammatory cytokines, may be considered. The results of our experiments with macrophage depletion using liposomal Cl$_2$MDP suggest that the observed increase in spleen size following dietary FO supplementation is largely due to accumulation of macrophages in the spleen. The immunohistochemical staining of spleen sections with B220 observations may partly explain the results obtained in the present study, since dietary supplementation with n-3 FA in mice basically leads to increased IL-1$\alpha$ and a decreased PGE$_2$ production capacity.
postendotoxin circulating TNF. It might be expected that Kupffer cells of the liver, due to their sinusoidal location, might be a major target for the uptake of liposomal Cl<sub>2</sub>MDP. In fact, recent evidence indicates that macrophage depletion in mice results in a 50 to 70% reduction in TNF mRNA in the liver following endotoxin challenge (46). Therefore, the Kupffer cells may be the most important contributors to the production of postendotoxin TNF. Similar to peritoneal macrophages, Kupffer cells may have increased TNF production capacity at the single cell level following dietary n-3 FA. We did not assess the number of Kupffer cells. The small increase in relative liver weight in FO-fed mice does not rule out a substantial increase in the number of Kupffer cells.

In conclusion, dietary n-3 FA supplementation in mice induces an aspecific increase in bone marrow cellularity, peripheral blood monocytes, accumulation of macrophages in the spleen, and increased postendotoxin circulating concentrations of TNF. The mechanism of these changes remains to be elucidated.

Acknowledgments

We thank Piet Spaan, Yvette Brom, and Reza Zadeh for technical assistance.

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8. Moore, F. A., E. E. Moore, K. A. Kudsk, R. O. Brown, R. H. Bower, M. J. Fa. We did not assess the number of Kupffer cells. The small increase in relative liver weight in FO-fed mice does not rule out a substantial increase in the number of Kupffer cells.

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