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# The Effect of Dietary Fatty Acid Manipulation on Phagocytic Activity and Cytokine Production by Peritoneal Cells from Balb/c Mice

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*Summary* Previous studies have demonstrated that dietary lipid manipulation may modify immune response by affecting lymphocyte proliferation, phagocytosis, cytokine production, etc. In this paper, we investigated the effect of olive oil (OO) on the phagocytic activity and cytokine production by murine peritoneal cells. These results were compared with those obtained from mice fed diets containing sunflower oil (SO) or hydrogenated coconut oil (HCO). Balb/c mice were divided into three groups and fed diets containing 15% by weight of either OO, SO or HCO for 5, 15, 30, 60 or 90 d. Phagocytic activity and interleukin-1 (IL-1) production were increased in OO-fed mice as compared to the other groups. On the contrary, no significant differences were observed in the levels of tumor necrosis factor (TNF) production, although the levels of this cytokine were slightly increased in mice fed the OO diet. These observations suggest that OO is able to modify the immune response and therefore, it may be used as an immunomodulatory agent.

***Key Words*** olive oil, sunflower oil, hydrogenated coconut oil, natural immunity

Dietary lipids or free fatty acids may modify the immune response by modulating immunological parameters such as phagocytosis (*1*, *2*) or cytokine production (*3*, *4*). Eicosapentaenoic and docosahexaenoic acids present in fish oils have been shown to play an important role in the management of chronic inflammatory disease states such as rheumatoid arthritis (*5*), psoriasis (*6*) and multiple sclerosis (*7*). Several studies have demonstrated that *n*-3 polyunsaturated fatty acids are capable of reducing neutrophil chemotaxis and leukotriene $B_4$ production in assays carried out in vivo as well as in vitro (*8*, *9*). These changes

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are not likely related to the modification of eicosanoid levels because it has been reported that the cyclooxygenase inhibitor indomethacin does not modify the chemiluminescence of peritoneal macrophages cultured in vitro with either arachidonic or eicosapentaenoic acids (*10*). Some studies have suggested that lipids may modulate the immune response by the alteration of membrane fluidity (*11*), production of lipid peroxides (*12*) and direct interaction with the cellular activation process (*13*).

Contradictory results have been reported concerning the effects of *n*-3 polyunsaturated fatty acid-containing diets on tumor necrosis factor (TNF) production. Studies carried out with mononuclear cells from human and Kupffer cells from rats showed a reduction of TNF production due to lipopolysaccharide (LPS) stimulation in fish oil-treated groups (*3*, *14*, *15*). On the contrary, similar diets were also shown to enhance TNF production (*16–18*). This fact may be explained by major differences in both methodological (duration and types of dietary supplementation) and biological factors such as species, age or sex (*19*).

The mechanism by which free fatty acids or oils added to a diet affect cytokine synthesis remains unclear. In assays performed in vitro, it has been shown that the inhibitory effect of unsaturated fatty acids is due to an independent mechanism of both eicosanoid synthesis and lipid peroxidation (*20*, *21*). However, a recent study suggests that these effects may be due to a direct regulatory effect on gene expression (*22*).

The purpose of this study was to analyse the immune response of peritoneal cells from mice fed diets containing each of the studied oils. We compare the effects of feeding mice a diet containing olive oil (OO) with the effects of the feeding of sunflower oil (SO) or hydrogenated coconut oil (HCO) diets. These effects may be due to components other than oleic acid contained in OO, such as alcohols, carotenoids, etc. In a recent study, Jeffery et al (*23*) have shown that the effects of an OO diet are most likely due to the presence of oleic acid, since rats fed a high-oleic sunflower oil diet showed identical effects as rats fed an OO diet, while these effects were different from those observed by the feeding of a SO diet.

Since phagocytic activity and cytokine production are involved in antitumour action and inflammatory states, oil-mediated modulation of these parameters may be useful to augment protection against infectious diseases and tumours.

## MATERIALS AND METHODS

*Animals and experimental diets.* Balb/c mice (obtained from colonies of our animal house) 10 weeks of age and 25–30 g of weight were housed in cages at a temperature of 24°C with a 12 h light-dark cycle and fed for 5, 15, 30, 60 or 90 d in each case prior to death. Animals were divided into three groups and each of them was allowed access, ad libitum, to water and one of the three experimental diets. Diets contained either (15% by weight) OO, SO or HCO. The HCO diet also contained 1% corn oil in order to prevent essential fatty acid

Table 1. Composition of the experimental diets.

| Ingredients | g/kg diet |
|---|---|
| Casein | 200 |
| D-L-Methionine | 3 |
| Corn starch | 315 |
| Sucrose | 205 |
| Cellulose fiber | 80 |
| Fats[1] | 150 |
| Mineral mix | 35 |
| Vitamin mix | 10 |
| Choline bitartrate | 2 |

[1] Assayed oils were olive oil (OO), sunflower oil (SO) and hydrogenated coconut oil (HCO).

deficiency. The composition of the diets is shown in Table 1.

*Isolation of mouse peritoneal cells.* Mice were sacrificed by cervical dislocation and the peritoneal cells were collected in Hank's balanced salt solution (HBSS) without phenol red (Sigma, St. Louis, MO, USA). The peritoneal cells were washed twice with HBSS at $200 \times g$ and 4°C for 5 min, and finally resuspended in HBSS for chemiluminescence assay, or in HEPES-buffered RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma), penicillin (50 U/mL) (Sigma), streptomicin (50 μg/mL) (Sigma), 1% L-glutamine (Sigma), 1% sodium pyruvate (Sigma), 5% sodium bicarbonate (Sigma) and 1% 2-mercaptoethanol (Sigma) for IL-1-producing cultures. Cell viability, assessed by trypan blue exclusion, was greater than 95%.

*Chemiluminescence assay.* This assay was developed according to the technique described by Moller-Larsen et al (*24*), slightly modified. Peritoneal cells were adjusted to a concentration of $10^5$ cells/mL with HBSS without phenol red, and 100 μL of cellular suspension was removed to scintillation vials. Subsequently, cells were activated by adding 20 μL of opsonized dark-adapted zymosan A (Sigma) adjusted at a concentration of 50 mg/mL. Finally, 70 μL of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma) at a concentration of 10 μg/mL was added to each tube. The phagocytic activity of peritoneal cells was measured by photon emission (LS-1801, Beckman Instruments, Irvine, CA, USA).

*Production and determination of IL-1.* A thymocyte costimulation bioassay was used for the measurement of soluble IL-1, according to the method described by Muegge et al (*25*). Briefly, the peritoneal cells in HEPES-buffered RPMI 1640 medium supplemented with 10% FCS were adjusted at a concentration of $2 \times 10^6$ cells/mL. One milliliter of the cellular suspension was added to each well of a 6-well flat-bottomed microtitre plate (Corning, NY, USA) as well as 1 mL of LPS from *Escherichia coli* serotype 026:B6 (Sigma) at a concentration of 20 μg/mL. The mixtures were incubated for 24 h at 37°C in a 5% $CO_2$ atmosphere.

Supernatants were finally collected and stored at −80°C until quantitative assay.

The thymus from 6 to 8-week-old mice were asseptically removed, homogeneized and washed in HBSS. Thymocytes were resuspended in HEPES-buffered RPMI 1640 medium supplemented with 10% FCS and 10 μg/mL phytohemagglutinin (PHA) (Sigma). The cellular suspension was adjusted at a concentration of $10^7$ cells/mL. IL-1 test samples were serially diluted 5-fold in complete medium without PHA. One hundred microliters of thymocyte suspension and 100 μL of diluted samples were added to each well of a 96-well flat-bottomed microtitre plate (Corning). The samples were incubated for 48 h at 37°C in a 5% $CO_2$ atmosphere. Cellular proliferation was measured by a colorimetric assay according to the method described by Mosmann (*26*). Finally, the absorbance was measured in a microplate reader (Whittaker Microplate Reader 2001, Salzburg, Austria) using a test wavelength of 550 nm and a reference wavelength of 620 nm. The values were expressed as optical density units.

*Production and determination of TNF-α.* TNF-α concentrations were measured by a specific enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Cambridge, MA, USA). Samples for this assay consisted of peritoneal cell supernatants obtained and stored as described for IL-1 production. The assay was performed according to the manufacturer's instructions, and the absorbance was measured by an ELISA microplate reader (Whittaker 2001) using a test wavelength of 450 nm and a reference wavelength of 550 nm. The values were expressed as optical density units.

*Statistical analysis.* Results are shown as means ± SE of cell preparations from five animals fed each of the diets. Statistical analysis was performed with the use of two-way factorial ANOVA, using Statgraphics statistical software version 5.0 (Statistical Graphics Corporation, STSC). Differences were considered statistically significant at $p < 0.05$.

## RESULTS

### *Mouse weight and peritoneal cell counts*

There were no significant differences in the body weights (Table 2) or counts of cells from the peritoneum of mice consuming the different diets over the 90-d feeding period (Fig. 1).

### *Measurement of phagocytic activity*

Figure 2 shows luminol-dependent chemiluminescence induced by the phagocytosis of peritoneal cells from mice fed OO, SO or HCO diets over the 90-d feeding period. From the 40th day, the values of activation from the peritoneal cells of OO-fed mice were higher than those obtained for SO- or HCO-fed mice. On the 90th day of OO diet supplementation, these differences were found to be statistically significant with regard to the values measured for mice fed SO or HCO diets ($p < 0.05$).

Table 2. Body weight of Balb/c mice fed experimental diets containing OO, SO or HCO for 90 d.

| Time (d) | Body weight (g) | | |
|---|---|---|---|
| | OO | SO | HCO |
| 5 | 26.3±0.7 | 25.9±0.8 | 26.5±0.4 |
| 15 | 27.0±0.3 | 26.8±0.5 | 27.8±0.4 |
| 30 | 27.6±0.4 | 27.8±0.2 | 28.9±0.3 |
| 60 | 28.2±0.4 | 28.1±0.2 | 29.3±0.4 |
| 90 | 28.4±0.6 | 28.9±0.1 | 30.6±1.0 |

Data are mean±SE from 5 animals. No statistically significant differences were found.

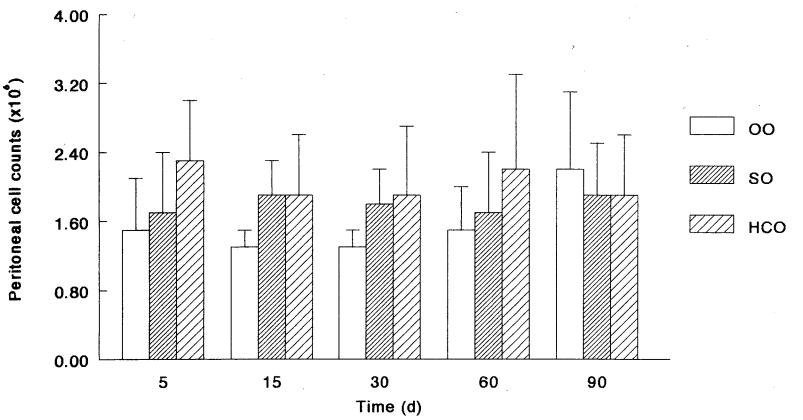


Fig. 1. Counts of peritoneal cells from mice fed diets containing OO, SO or HCO for 90 d. Data are mean±SE of cell preparations from 5 animals. No statistically significant differences were found.

*Determination of IL-1*

IL-1 production by LPS-stimulated peritoneal cells obtained from the different dietary groups is illustrated in Fig. 3. Although cells from the HCO-fed group displayed the lowest levels of this cytokine, we did not find significant differences between the SO- and HCO-fed mice except on the 15th day of dietary lipid supplementation. On the contrary, IL-1 production in OO-fed mice was greater than in the other groups studied. Statistical analysis showed significant differences ($p<0.05$) between the OO-fed group and other groups during the time periods analysed, except for the 30th day of dietary fat supplementation.

*Determination of TNF-α*

Figure 4 shows the TNF-α production by LPS-stimulated peritoneal cells isolated from mice fed experimental diets. Results show that TNF production in

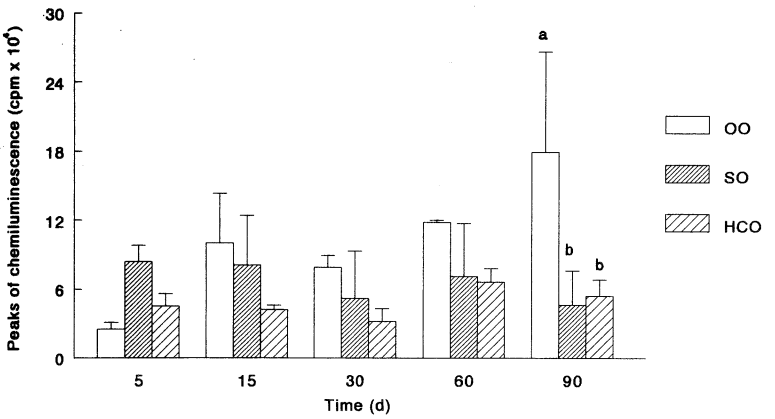


Fig. 2. Peaks of chemiluminescence from peritoneal cells of mice fed diets containing OO, SO or HCO for 90 d. Data are mean ± SE of cell preparations from 5 animals expressed as counts per minute (cpm). Results in a row with different superscript are significantly different at $p < 0.05$.

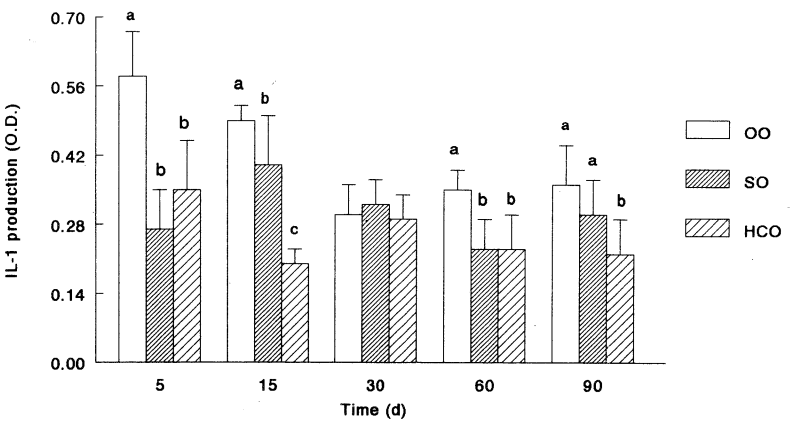


Fig. 3. IL-1 production in mice fed diets containing OO, SO or HCO for 90 d. Data are mean ± SE of cell preparations from 5 animals expressed as optical density units (O.D.). Results in a row with different superscript are significantly different at $p < 0.05$.

OO-fed mice was greater than that in SO- or HCO-fed mice, whereas the smallest values of TNF secretion were found in HCO-fed mouse-derived cells. However, these differences were not statistically significant.

## DISCUSSION

In this study we investigated the effect of a diet containing OO on the cytokine production and phagocytic activity of peritoneal cells obtained from mice fed for

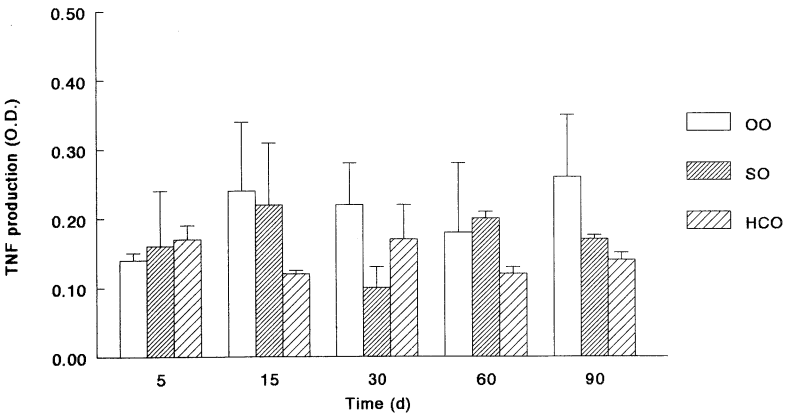


Fig. 4. TNF-α production in mice fed diets containing OO, SO or HCO for 90 d. Data are mean ± SE of cell preparations from 5 animals expressed as optical density units (O.D.). No statistically significant differences were found.

5, 15, 30, 60 or 90 d. These results have been compared with those obtained from mice fed diets containing SO or HCO. The major proportion of the oils contained in the diets consisted of monounsaturated fatty acids (OO), polyunsaturated fatty acids (SO) or saturated fatty acids (HCO).

The results from this study show that dietary lipid manipulation modifies several immune parameters of murine peritoneal cells. There were no significant differences in weight gain between animals fed the different diets. Peritoneal cell numbers from mice consuming different diets were similar. However, the phagocytic activity of peritoneal cells obtained from OO-fed mice was significantly greater than that in the SO or HCO-fed mice. Moreover, peritoneal cells obtained from the OO-fed mice also produced more IL-1 and TNF-α than those isolated from the other groups.

Monocyte functions such as phagocytosis are altered by dietary lipids. Several studies have reported that unsaturated fatty acids incorporated to assays in vitro increase the phagocytosis of zymosan particles (*11*, *27*). These studies showed a high correlation between phagocytosis and membrane polyunsaturated fatty acid content. The results reported here suggest another mechanism for the action of unsaturated fatty acids, likely related to the index of unsaturation. The observations of our study support the idea that an OO diet increases the phagocytosis of zymosan particles, while saturated fats such as HCO cause the lowest rate of phagocytosis. On the other hand, the results of chemiluminescence obtained using peritoneal macrophages from rats fed a diet containing eicosatetraenoic acid were approximately half the value of the chemiluminescence found in macrophages cultured with eicosapentaenoic acid (*10*).

IL-1 and TNF are cytokines involved in the pathogenesis of inflammatory diseases. It has been demonstrated that the supplementation of *n*-3 polyunsaturated

fatty acids suppresses the capacity of mononuclear cells to synthesize IL-1 and TNF-α (*3*). This fact may contribute to the amelioration of inflammatory symptoms in patients who suffer from these diseases. Tappia and Grimble (*28*) reported that OO has immunosuppressive activity because it has anti-inflammatory effects, and Linos et al (*29*) suggested that populations who consume large amounts of OO have a lower incidence of rheumatoid arthritis. Our results are consistent with Tappia and Grimble (*28*), since these authors described a slight reduction in TNF production from peritoneal macrophages of rats fed a diet containing OO after 8 weeks. However, this reduction did not show significant differences. Our data do not confirm a reduction, since we observed a slight increase in TNF production. However, we did not find significant differences among the groups studied. Our results concerning TNF production are also in accordance with Chang et al (*17*), who showed that TNF production from Swiss Webster mice was increased by fish oil diets and reduced by diets which contained HCO or corn oil. Hardardóttir and Kinsella (*4*) observed an increase in TNF production when the ratio of *n*-3/*n*-6 polyunsaturated fatty acids was increased from 0 to 1. Turek et al (*30*) also observed an increase in TNF production by macrophages from mice consuming diets which contained *n*-3 polyunsaturated fatty acids. Therefore, an OO diet may be able to modulate TNF-α production like *n*-3 polyunsaturated fatty acids.

However, several studies carried out using both humans and animals have shown that TNF and IL-1 production are reduced by fish oil-containing diets (*3*, *14*, *15*). These disparities could be explained by differences in cell populations and the species used in the experimental assays (*14*). On the other hand, the use of *n*-3 and *n*-6 PUFA-containing diets showed that the modification in the cytokine levels may be related to the increased *n*-3 and decreased *n*-6 fatty acid concentrations in the peritoneal cell membrane (*4*). This mechanism may be responsible for the inhibition of the ciclooxygenase pathway, reduction of prostaglandin synthesis and subsequent increase in TNF production induced by fish oil.

Other studies have demonstrated that dietary *n*-3 fatty acids do not inhibit, but significantly increase the IL-1 production in mouse peritoneal macrophages (*18*). Our results about IL-1 production indicate a significant reduction before 60 d and an increase from 60 d to the end of supplementation with the diet containing OO. These data are in agreement with those from Tappia and Grimble (*28*), who demonstrated a significant reduction in IL-1 production in the peritoneal macrophages of rats after 4 weeks, as well as an increase in this cytokine after 8 weeks of supplementation with a diet containing OO.

IL-1 and TNF-α production are modulated by dietary lipids, while in humans, *n*-3 polyunsaturated fatty acids reduce cytokine production. In animal models, the results are contradictory because Billiar et al (*14*) demonstrated that both IL-1 and TNF production are reduced by *n*-3 fatty acids in the Kupffer cells of rats. On the contrary, other studies have reported that IL-1 and TNF production increase after dietary lipid manipulation (*16*, *18*, *31*). These discrepancies in animal models may be due to differences in the cell population studied or in the animal species used

for the assays, although this aspect is not clear yet.

In short, the results obtained show that a diet containing OO enhances several parameters involved in the inflammatory process, such as the peaks of phagocytic activity and the production of IL-1 and TNF. Moreover, these effects are likely due to oleic acid rather than to other components of OO, as a recent study confirms (*23*). Statistical analyses have also indicated that IL-1 production is time-dependent. Perhaps, this effect may be due to the number of peritoneal cells, which began to increase from 60 d of supplementation with the diet containing OO; in the same way that IL-1 production augments. On the contrary, statistical analyses have shown that neither peaks of chemiluminescence nor TNF production are time-dependent.

Therefore, dietary lipid manipulation may be useful to increase resistance to infection as well as for immune surveillance against tumour cells. Further studies should be performed to understand the mechanisms involved in the immune response regulation by fatty acids contained in oils.

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