Assessment of Interleukin-12, Gamma Interferon, and Tumor Necrosis Factor Alpha Secretion in Sera from Mice Fed with Dietary Lipids during Different Stages of *Listeria monocytogenes* Infection

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Received 22 December 2004/Returned for modification 3 February 2005/Accepted 22 June 2005

Recent experimental observations have determined that long-chain *n*-3 polyunsaturated fatty acids suppress immune functions and are involved in the reduction of infectious disease resistance. BALB/c mice were fed for 4 weeks with one of four diets containing either olive oil (OO), fish oil (FO), hydrogenated coconut oil, or a low fat level. Interleukin-12p70 (IL-12p70), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) production in the sera of mice fed these diets and challenged with *Listeria monocytogenes* were determined by enzyme-linked immunosorbent assay. In addition, bacterial counts from spleens of mice were carried out at 24, 72, or 96 h of infection. Here, we quantified an initial diminution of production of both IL-12p70 and IFN-γ, which appear to play an important role in the reduction of host resistance to *Listeria monocytogenes* infection. In addition, an efficient elimination of *Listeria monocytogenes* was observed in spleens of mice fed a diet containing OO at 96 h of infection, despite reductions in IL-12p70 and TNF-α production, suggesting an improvement of immune resistance. Overall, our results indicate that the initial reduction of both IL-12 and IFN-γ production before *Listeria monocytogenes* infection represents the most relevant event that corroborates the impairment of immune resistance by *n*-3 polyunsaturated fatty acids during the different stages of infection. However, we speculate that the modulation of other cytokines must be also involved in this response, because the alteration of cytokine production in mice fed an FO diet in a late phase of *Listeria monocytogenes* infection was similar to that in mice fed OO, whereas the ability to eliminate this bacterium from the spleen was improved in the latter group.

Evidence from numerous investigations has revealed that nutritional status is a crucial factor that contributes to the modulation of the host immune response (6). Hence, the interactions between certain nutrients and immunity play a relevant role that should be evaluated from biological and clinical points of view (11). In the last few years, a large number of epidemiological, clinical, and experimental studies have indicated that certain dietary lipids are capable of altering different immune functions in both animals and humans (5, 9). Accordingly, diets containing moderate levels of oily fish (mainly constituted by long-chain *n*-3 polyunsaturated fatty acids) exert numerous health benefits, such as reduction of autoimmune disorders (17, 18) or diminution of cancer incidence (25). As a direct consequence, different investigations have applied several fatty acids in clinical nutrition, because long-chain *n*-3 polyunsaturated fatty acids derived from fish oil have been demonstrated to be more immunosuppressive than *n*-6 polyunsaturated fatty acids or *n*-9 monounsaturated fatty acids. Despite this, several studies have reported that an excess intake of *n*-3 polyunsaturated fatty acids may cause undesirable effects in both humans and animals, because they are able to reduce host immune resistance against infectious microorganisms (1, 11). Thus, numerous investigations have indicated that diets containing *n*-3 polyunsaturated fatty acids enhance susceptibility to infection by *Listeria monocytogenes* (8, 13, 23), *Salmonella enterica* serovar Typhimurium (7), *Mycobacterium tuberculosis* (21), or *Pseudomonas aeruginosa* (22), whereas other recent observations have reported that these diets increase immune resistance against *Klebsiella pneumoniae* (2, 3, 27), although these effects could not be demonstrated in infection with *Streptococcus pneumoniae* (27). Therefore, it is possible that the consequences derived from the reduction of immune resistance by *n*-3 polyunsaturated fatty acids may also depend on the type of microorganisms and their pathogenic mechanisms. Based on the exposed arguments, it is important to consider the factors involved in the reduction of immune resistance caused by many dietary lipids. In fact, cytokines are important immune mediators that participate in the development of immune responses. Thus, production of interleukin-12p70 (IL-12p70), an inflammatory cytokine that plays a critical role in the Th1-type response, is reduced after fish oil diet administration (13, 24), whereas production of IL-4 (an anti-inflammatory cytokine) is increased, although the alteration in the levels of this cytokine does not appear to constitute a critical factor responsible for the reduction of immune defense after dietary lipid administration (24). Nevertheless, other studies have indicated that fish oil diets reduce the secretion of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1, or IL-6 (26), and therefore this leads to an increase in percent survival after lipopolysaccharide injection (20). We and others have suggested that the reduction of cytokine synthesis caused by *n*-3 polyunsaturated fatty acids indicates that these fatty acids could affect the host response to
bacterial infections (10, 11, 24). As a direct consequence of these effects, a reduction in percent survival of mice as well as an inefficient ability to eliminate the microorganisms from liver and spleen has been reported (8, 13).

Here, we have focused on the evaluation of IL-12p70, gamma interferon-γ (IFN-γ), and TNF-α production after the administration of dietary lipids in mice infected with *L. monocytogenes*, which has established an important model system for understanding host resistance to intracellular bacteria. The measurement of the cytokines in sera from mice fed with dietary lipids was carried out during different stages of murine listeriosis.

### MATERIALS AND METHODS

**Animals and experimental diets.** BALB/c mice 8 to 10 weeks old (from a breeding colony of the research technical services from the University of Jaén) were allocated to receive one of four diets for 4 weeks, and each group had unlimited access to food and water during the experiment. Mice were housed in cages in an environmentally controlled room at a temperature of 24°C with a 12-h light-dark cycle. Diets contained either olive oil (OO) (20%), fish oil (FO) (20%), or hydrogenated coconut oil (HCO) (20%). In addition, another group was fed a diet containing a low fat (LF) (2.5% of fats) level and was used as a control. The diet containing HCO was supplemented with 1% corn oil in order to prevent essential fatty acid deficiency. The compositions of the experimental diets are illustrated in Table 1.

**Bacterial growth, experimental infection, and serum collection.** *L. monocytogenes* was grown in blood tryptic soy agar medium (Scharlau Chemie, Barcelona, Spain) for 24 h at 37°C. The concentration of the cell suspension was adjusted spectrophotometrically at 550 nm. At the end of dietary lipid administration, each mouse was infected with a virulent strain of *L. monocytogenes* injected through the tail vein (10⁵ CFU/ml), and peripheral blood was isolated at 0, 24, 72, and 96 h after infection. Bacterial counts were determined as described in Materials and Methods. The number of bacterial colonies was counted, and the results are expressed as log₁₀ viable bacteria. The bars represent mean values ± SEM for three independent determinations (n = 5 in each dietary group). *P < 0.05 compared with the values from the control group.

### RESULTS

**Quantification of bacterial loads in spleens of mice.** After the administration of dietary lipids, *L. monocytogenes* was inoculated in each mouse. We quantified the number of viable bacteria isolated from spleens of mice experimentally infected with *L. monocytogenes* and thus determined the capacity of the host immune response to eliminate this bacterium from spleens of mice fed dietary lipids. Figure 1 shows a statistically significant increase of viable bacteria from spleens of mice fed a diet containing FO at 24, 72, and 96 h after experimental infection (*P < 0.05*). In contrast, the group fed a diet containing FO was the most efficient in *L. monocytogenes* elimination.

**IL-12p70 production.** It is well established that cytokines play a crucial role in the regulation of the immune system, but their production is also modulated after dietary lipid administration. IL-12 is an important cytokine that participates in the cellular immune response to intracellular microorganisms such as *L. monocytogenes* infection was determined at 0, 24, 72, and 96 h. Absorbance at 405 nm was read using a Microplate reader (Whittaker 2001, Salzburg, Austria). Results were calculated against standard curves generated using known amounts of recombinant cytokines. The limits of detection of these assays were <2.5 pg/ml (IL-12p70), <5.1 pg/ml (TNF-α), and <2 pg/ml (IFN-γ).

**Statistical analysis.** Results are presented as means ± standard errors of the means (SEM). Data were determined by analysis of variance to compare the effects of experimental and control (LF) diets. When analysis of variance indicated significant differences, the treatment means were compared using Fisher’s least-significant difference test. Differences were considered statistically significant at a *P* value of <0.05.

### TABLE 1. Compositions of the experimental diets

<table>
<thead>
<tr>
<th>Component</th>
<th>g/kg diet</th>
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<tbody>
<tr>
<td>Casein</td>
<td>200</td>
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<tr>
<td>dl-Methionine</td>
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<tr>
<td>Cornstarch</td>
<td>315</td>
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<tr>
<td>Sucrose</td>
<td>155</td>
</tr>
<tr>
<td>Fiber</td>
<td>80</td>
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<tr>
<td>Fat</td>
<td>200</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
</tr>
<tr>
<td>Choline</td>
<td>2</td>
</tr>
</tbody>
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* a BALB/c mice were fed their respective diets for 4 weeks.

b Oils incorporated in the diets were OO, FO, or HCO. The control group was fed a diet containing 2.5% lipid by weight (LF diet).

**Determination of numbers of viable *L. monocytogenes* in spleens.** Mice were infected with 100 μl of a *L. monocytogenes* cell suspension (10⁷ CFU/ml) and sacrificed at 24, 72, and 96 h postinfection. Spleens were removed under sterile conditions in phosphate-buffered saline (pH 7.4). Spleens were then prepared by being homogenized between frosted-glass slides in distilled water in order to release intracellular bacteria at 24, 72, and 96 h after infection with the bacteria. Serial 10-fold dilutions of each sample were incubated on blood tryptic soy agar medium to determine the number of viable *L. monocytogenes* organisms. Finally, plates were incubated at 37°C for 24 h, and the number of bacterial colonies was counted. Results were expressed as the log₁₀ viable bacteria in spleens.

**Quantification of cytokine levels in serum by immunoassay.** IL-12p70, IFN-γ, and TNF-α secretion were measured by enzyme-linked immunosorbent assay (ELISA) (assay kits were from R&D Systems, Minneapolis, MN) according to the manufacturer’s protocols. Cytokine synthesis during different stages of *L. monocytogenes* infection was determined at 0, 24, 72, and 96 h. Absorbance at 405 nm was read using a Microplate reader (Whittaker 2001, Salzburg, Austria). Results were calculated against standard curves generated using known amounts of recombinant cytokines. The limits of detection of these assays were <2.5 pg/ml (IL-12p70), <5.1 pg/ml (TNF-α), and <2 pg/ml (IFN-γ).

**Statistical analysis.** Results are presented as means ± standard errors of the means (SEM). Data were determined by analysis of variance to compare the effects of experimental and control (LF) diets. When analysis of variance indicated significant differences, the treatment means were compared using Fisher’s least-significant difference test. Differences were considered statistically significant at a *P* value of <0.05.
as *L. monocytogenes*. Our results reveal that IL-12p70 production from mice fed diets containing OO or FO was significantly reduced at 72 and 96 h after experimental infection with *L. monocytogenes*, compared with values for the control group (*P* < 0.05) (Fig. 2B and C). In addition, an initial significant reduction of IL-12p70 production was observed in the sera of mice fed an FO diet. In contrast, IL-12p70 secretion from mice fed a diet containing HCO was not altered with respect to values for the control group (Fig. 2D).

**IFN-γ production.** Similarly, IFN-γ production is crucial to an efficient immune response to this intracellular pathogen. Here, IFN-γ secretion was significantly increased in the groups fed diets containing OO, FO, or HCO at 96 h of experimental infection compared with data from the LF group (*P* < 0.05) (Fig. 3B, C, and D). However, a statistically significant reduction of IFN-γ production was observed in the group fed a diet containing FO before experimental infection with *L. monocytogenes* (*P* < 0.05) (Fig. 3C).

**TNF-α production.** To evaluate the action of one of the most important proinflammatory cytokines, we analyzed the production of TNF-α from sera of mice fed dietary lipids. These results demonstrate a significant enhancement of TNF-α secretion from mice fed diets containing OO or FO before and at 24 h of experimental infection with *L. monocytogenes* (*P* < 0.05) (Fig. 4B and C). However, TNF-α secretion was significantly reduced at 72 or 96 h of experimental infection with *L. monocytogenes* in the groups fed diets containing OO or FO (*P* < 0.05), although the production of this proinflammatory cytokine was very similar at each time point. In contrast, an enhancement of TNF-α production was observed at 96 h of infection in the groups fed diets containing LF or HCO with respect to values before experimental infection with *L. monocytogenes* (Fig. 4A and D). In fact, the kinetics of TNF-α production were similar in these two groups, with the exception of a significant increase in the HCO group at 96 h of infection with *L. monocytogenes*.

**DISCUSSION**

The investigation of the events that occur during the infectious processes contributes to establishment of the action of dietary lipids on the immune system in the course of an infection promoted by *L. monocytogenes*. Numerous studies have indicated that the alteration in secretion of different cytokines by the action of certain dietary lipids constitutes a critical factor responsible for the modulation of immune and inflammatory responses (5, 9, 32). Therefore, the relationship between dietary lipids and their role in the reduction of the host immune defense against infectious agents acquires substantial importance.

It is commonly recognized that protective immunity to intracellular bacteria, such as *L. monocytogenes*, requires a co-
Ordinated interaction between many cell types and the production of numerous cytokines (12). These responses are efficiently synchronized by the proinflammatory cytokine IL-12, which promotes T-cell differentiation toward a cell-mediated (T-helper 1-type) immune response (28). Indeed, IL-12 is a crucial cytokine for innate and adaptive immunity, contributing to the alteration of the T-cell repertoire after infection with intracellularly growing pathogens (4, 16). In addition, IL-12 and TNF-α, two crucial cytokines of the innate immune system, synergize to cause NK cell secretion of IFN-γ, leading to macrophage activation. Thus, neutralization of IL-12, TNF-α, or IFN-γ demonstrated the critical role that these cytokines play during the infection with L. monocytogenes (19, 30), such that mice lacking any of these cytokines were unable to protect themselves against an overwhelming infection (31).

As mentioned above, the potential action of several dietary lipids on the modulation of immune system functions (5, 9), and particularly on the alteration of cytokine production (32), has been widely demonstrated. In order to elucidate the action of diverse dietary lipids on host immune resistance, the present study evaluated the effects of diets containing OO, FO, or HCO on IL-12p70, IFN-γ, and TNF-α production during different stages of L. monocytogenes infection.

Previous investigations have determined that diets containing FO show beneficial effects in the reduction of inflammatory disorders, which leads to a decrease of autoimmune disease incidence (17). Nevertheless, this characteristic may be overshadowed by an impairment of natural host resistance after the administration of an FO diet, as a consequence of exacerbated immunosuppression (8, 13). Thus, results from our group and others have determined that the administration of a diet containing FO is associated with a reduction of survival percentage as well as an increase in the number of viable bacteria from spleens and livers of mice challenged with L. monocytogenes, suggesting an impairment of the host immune defense (8, 13). Therefore, it is important to explain the reasons why the host immune response is reduced after the administration of certain dietary lipids. Previously, some interesting reports from Fritsche and coworkers suggested that a reduction of both IL-12 and IFN-γ secretion at 24 h postchallenge plays a relevant role in the impairment of immune resistance in mice fed an FO diet (14, 15). However, irrespective of the relevance of an FO diet in the alteration of immune system functions, it is important to determine the immunomodulation exerted by other fats during different stages of L. monocytogenes infection. Thus, a diet containing OO appears to reduce IL-12p70 production, as well as to significantly decrease the secretion of TNF-α at 72 or 96 h of experimental infection with L. monocytogenes, but it is important to emphasize that this diet is not involved in an initial reduction of either IL-12p70 or IFN-γ production. Despite the significant reduction of IL-12p70 and TNF-α production at 96 h of experimental infection in the group fed an OO diet, an efficient elimination of L. monocytogenes from spleen is exhibited. It is probable that the absence of changes in the production of these cytokines before infection with L. monocytogenes is responsible in part for these effects, whereas the initial reduction of IL-12p70 and IFN-γ production in the FO group is associated with a considerable increase in the number of viable bacteria from spleens. The initial reduction of IFN-γ production in the FO group constitutes an important result, because no production of this cytokine was seen in the LF and HCO groups at 96 h of experimental infection, whereas the number of L. monocytogenes organisms from spleen varied considerably between the FO and LF groups or the FO and HCO groups. Hence, this result confirms again our preliminary argument, which is supported by the participation of other types of cytokines which should be modulated by dietary lipids.

Our recent observations have indicated that the type of dietary lipids may affect circulating concentrations of IL-12p70. This event along with inappropriate action of IL-4 contributes to an impairment of host immune susceptibility to infection (24). Here, the reduction of IL-12p70 levels after experimental infection with L. monocytogenes gave rise to an important diminution of IFN-γ secretion in the four groups studied; however, this reduction is more accentuated in the LF and HCO groups at 96 h of infection. Instead, other investigations have underscored that the depletion of IL-12 decreases IFN-γ production and exacerbates infection in intracellular infection (29). Our results have demonstrated that despite the reduction of both IL-12p70 and IFN-γ production at 96 h of experimental infection, a diminution in the number of viable bacteria from spleens of mice occurs in the four groups. Hence, we can verify that the increase in the number of viable bacteria from spleens of mice fed with an FO diet may be attributed to an initial
reduction of both IL-12p70 and IFN-γ secretion, whereas the reduction of viable bacteria from spleens of mice observed at 96 h after experimental infection with *L. monocytogenes* in the four groups could be related to an increase in TNF-α production. In short, although a reduction of viable bacteria has been observed at 96 h of infection, an inefficient resolution of the infectious process has been detected in the group fed a diet containing FO. Thus, the administration of an FO diet does not improve the host immune resistance during the different stages of *L. monocytogenes* infection. However, we speculate that other cytokines must be also involved in this response, because the alteration of cytokine production in the FO group is similar to that in the group fed with an OO diet (with the particular exception of a significant reduction of both IL-12p70 and IFN-γ initially observed in the group fed a diet containing FO), whereas the number of viable bacteria from spleens is considerably reduced in the OO group, indicating an improvement of immune resistance by the generation of protective immunity.

**Concluding remarks.** The present results confirm that the reduction of host immune resistance to infectious microorganisms could be particularly associated with an initial decrease of both IL-12p70 and IFN-γ secretion in animals challenged with *L. monocytogenes* after the administration of a diet containing FO. The reduction of proinflammatory cytokine levels produces a diminution in macrophage activation that is responsible for an inefficient ability to eliminate *L. monocytogenes*. Nevertheless, in spite of the fact that an important increase of IFN-γ secretion was observed in the group fed a diet containing FO in a late stage of *L. monocytogenes* infection (72 and 96 h) with respect to the group fed an LF diet, a relevant impairment of the immune defense has been widely described (8, 13) (Fig. 5). Recent experimental observations have shown that it is probable that the reduction of IFN-γ receptor expression participates in part in the inefficient elimination of *L. monocytogenes* after FO diet administration (14, 15). In addition to this important consideration, we suggest that the modulation of other cytokines by the action of fatty acids contained in FO may constitute a critical factor directly involved in the capacity of immune cells to eliminate this pathogen.

Overall, we hypothesize that the coordinated action of these and others cytokines represents an important aspect that should be analyzed in further studies to demonstrate the potential action of diets containing unsaturated fatty acids on the host immune defense against infectious microorganisms.

**ACKNOWLEDGMENTS**

This study was supported by grants from the Department of Education and Science (Autonomous Government of Andalusia, Spain, Group CT105), the Ministry of Science and Technology (grant BSA2001-3648), and the University of Jaén. M. A. Puertollano receives a fellowship from the Teaching and Research Staff Training Programme (Department of Education and Science, Autonomous Government of Andalusia, Spain). E. Puertollano receives a fellowship from the Ministry of Education and Science, Spain.

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