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Effect of Dietary Oils on Host Resistance to Fungal Infection in Psychologically Stressed Mice

Motoko OARADA,[1,†] Miki IGARASHI,[2] Tsuyoshi TSUZUKI,[3] Nobuyuki KURITA,[1] Tohru GONOI,[1] Takeshi NIKAWA,[4] Katsuya HIRASAKA,[4] Teruo MIYAZAWA,[2] Kiyotaka NAKAGAWA,[2] and Katsuhiko KAMEI[1]

[1]*Medical Mycology Research Center, Chiba University, Chiba 260-8673, Japan*
[2]*Biodynamic Chemistry, Graduate School of Life Science and Agriculture, Tohoku University, Sendai 981-8555, Japan*
[3]*Food and Biomolecular Science, Graduate School of Life Science and Agriculture, Tohoku University, Sendai 981-8555, Japan*
[4]*Department of Nutrition, Tokushima University School of Medicine, Tokushima 770-8503, Japan*

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Psychological stress can modulate host defense against invading pathogens. In this study, we investigated the effect of dietary oils on social isolation stress-induced modulation of host resistance to *Paracoccidioides brasiliensis*. In olive oil-fed mice, 3 weeks of isolation stress resulted in temporarily delayed clearance of this fungus in the liver compared with group-housed mice. By contrast, in soybean oil-fed mice, isolation stress had no significant effect on antifungal activity. The olive oil-fed mice showed greater liver interferon (IFN)-γ and interleukin (IL)-6 production in response to infection as compared with the soybean oil-fed mice. In the olive oil-fed mice, isolation stress led to greater infection-induced IFN-γ production in the liver compared with the group-housed animals. These results indicate that the modulatory effects of psychological stress on host resistance to *P. brasiliensis* can vary depending on dietary fatty acid composition.

Key words: antifungal activity; dietary olive oil; dietary soybean oil; interferon (IFN)-γ; social isolation stress

Psychological stress has been reported to modify multiple aspects of immune responses.[1,2] For example, social isolation stress decreases natural killer (NK) cell activity and enhances the metastasis of transplantable tumors in mice.[3] In our previous study, 2 weeks of social isolation stress reduced mitogen-stimulated lymphocyte proliferative responses and altered cytokine production by lymphocytes.[4] These isolation stress-induced modulations were more profound in olive oil-fed mice than soybean oil- or fish oil-fed mice. It has been reported that psychological stress can modulate the fatty acid composition of total lipids in liver, lymphocytes, and serum.[4–6] Our previous study showed that isolation stress reduced the arachidonic acid (AA) content of lymphocytes markedly, moderately, and not at all in olive oil-, soybean oil-, and fish oil-fed mice respectively.[4] AA is precursor of prostaglandins, leukotrienes, and related compounds, which have important roles in inflammation and in the regulation of immunity.[7] It has been reported that a decrease in the AA content of immunocompetent cells is accompanied by reductions in lymphocyte proliferation, inflammatory cytokine production, and NK cell activity.[7–9] Thus, the isolation stress-induced greater modulation of lymphocyte immunological activities observed with olive oil may have been due, at least partly, to a marked decrease in the arachidonic acid content of the lymphocytes. It is also well accepted that as a consequence of the modulatory effects of psychological stress on immune responses, psychological stress can modulate host defenses against invading pathogens and can alter susceptibility to infectious diseases.[10,11] Adult mice that as pups experienced handling with maternal separation exhibit increased susceptibility to influenza viral infection.[12] In an attempt to determine whether dietary oils with different fatty acid compositions can differently modulate psychological stress-induced alteration of host resistance, we further investigated the effects of dietary oils (olive oil and soybean oil) on host resistance to *P. brasiliensis* under conditions of isolation stress in mice.

Materials and Methods

Experimental diets. The experimental diets used in this study were designed according to the AIN-76A diet guidelines,[13] with minor modifications as necessary to accommodate an increase in caloric density as the fat content increased from 5% to 11% by weight. Casein, α-corn starch, sucrose, cellulose powder, AIN-76A mineral mixture, AIN-76A vitamin mixture, and choline bitartrate were purchased from Oriental Yeast (Tokyo). DL-methionine was from Wako Pure Chemical (Osaka, Japan). Olive oil and soybean oil were kindly supplied by NOF (Tokyo). The test diet (in wt.%) consisted of 21.5% casein, 46.8% α-corn starch, 10% sucrose, 5.35% cellulose powder, a 3.75% AIN-76A mineral mixture, a 1.07% AIN-76A vitamin mixture (containing 5 mg/g of *dl*-α-tocopherol acetate), 0.32% DL-methionine, 0.21% choline bitartate, and 11.0% test oil. The two diet groups used in our analyses were differentiated by their lipid sources (in wt.%): 11.0% olive oil and 11.0% soybean oil. Table 1 presents the fatty acid composition of the various dietary oils. Prior to the addition of the dietary oils to the basic fat-free mixture, all-*rac*-α-tocopherol (Wako) was mixed with the various oil to a final concentration of 12 mg of α-tocopherol/100 g the oil.

† To whom correspondence should be addressed. Tel: +81-43-226-2787; Fax: +81-43-226-2486; E-mail: motoko.o@faculty.chiba-u.jp
Abbreviations: AA, arachidonic acid; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; NK, natural killer

Fungi. P. brasiliensis isolate Pb-18, isolated from a Brazilian patient with paracoccidioidomycosis, was used to infect the mice. The fungal cells used in the experiments were newly derived from the mycelial form of this microbe and were subcultured twice at 35 °C at 4-d intervals on 1% glucose-supplemented brain heart infusion (BHI; Difco Laboratories, Detroit, MI) agar slants. Fresh growth (4 d) of the fungus was collected in 0.9% sterile saline with mesh to eliminate cell clumps. After they were washed once, the fungal cells were counted using a hemacytometer. More than 97% of the fungal cells prepared in this manner were viable, and these were resuspended at the indicated densities in saline prior to infecting the mice.

Animal maintenance. Pathogen-free four-week-old female BALB/c mice were obtained from Charles River (Atsugi, Japan). The animals were group-housed (four mice per $32 \times 22 \times 11\,cm^3$ aluminum cage) and maintained on a standard non-purified diet (Oriental Yeast) and water *ad libitum* for 5 d, and then fed a test diet containing either 11% olive oil or soybean oil and water *ad libitum*. Nine d after commencement of these test diets, the mice were separated into two different groups that were (i) housed under the same conditions (four mice per $32 \times 22 \times 11\,cm^3$ aluminum cage, group-housed) or (ii) housed individually (one mouse per $30 \times 18 \times 11\,cm^3$ aluminum cage, isolated), and maintained on the same test diet and water. In both groups, a 12-h: 12-h light/dark cycle was maintained, and the room temperature was kept at 23 ± 1 °C. After 3 further weeks under these housing conditions, the animals were infected using a single intravenous injection of a 200 µl fungal cell suspension (9×10^6 cells/ml) *via* the lateral tail vein. The dose of *P. brasiliensis* (Pb-18) used in the present study (1.8×10^6 cells/mouse) was not lethal. The mice were sacrificed by decapitation on days 0–8 post-infection, and liver and spleen were harvested. All of the housing, handling and sample collection procedures described herein conformed to the policies and recommendations of the Laboratory Animal Care Advisory Committee of Chiba University.

Table 1. Fatty Acid Compositions of Dietary Oils (g/100 g of fatty acid)

Fatty acid	Olive oil	Soybean oil
16:0	12.6	17.4
16:1(n-9)	1.0	0.1
18:0	3.1	5.7
18:1(n-9)	75.6	22.3
18:1(n-7)	2.2	1.2
18:2(n-6)	4.5	46.9
18:3(n-3)	0.6	6.1
20:1(n-9)	0.2	0.2
20:4(n-6)	0.1	0.0
22:5(n-6)	0.1	0.0
22:5(n-3)	0.0	0.1
Total saturated fatty acids	15.7	23.1
Total MUFA	79.0	23.8
Total n-6 PUFA	4.7	46.9
Total n-3 PUFA	0.6	6.2
Total n-6+n-3 PUFA	5.3	53.1

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

Counts of viable fungal cells from organs. Spleens and livers were aseptically removed from the subject mice and homogenized in a glass homogenizer with 4 ml and 19 ml saline respectively. The samples were diluted with saline. One ml of each diluted homogenate was then plated on BHI agar supplemented with 50 µmol/l of EDTA, 20 ml/l of horse serum (Gibco Laboratories, Grand Island, NY) and 150 µmol/l of chloramphenicol (Wako). The agar plates were incubated in a humidified atmosphere at 35 °C, and the colony-forming units (CFU) of *P. brasiliensis* were counted after 21 d of incubation.

Measurement of IFN-γ and IL-6 levels. Mouse spleens and livers were homogenized in ice-cold PBS (10%, v/v) with a glass hand-held tissue homogenizer and centrifuged at 10,000 rpm for 30 min at 4 °C to pellet the debris. The levels of IFN-γ and IL-6 were determined in sample supernatants using ELISA kits purchased from Endogen (Rockford, IL). All assays were performed according to the manufacturer's instructions.

Statistical analysis. All data were expressed as mean plus standard deviation (SD) for *n* observations. Data were analyzed by Tukey's test after two-way ANOVA with SPSS software (SPSS, Tokyo). Differences were considered significant at $p < 0.05$.

Results

Effects of dietary oils and isolation stress on liver and spleen weights of P. brasiliensis*-infected mice*

In all groups, the liver and spleen weights increased in response to infection. The liver weight in the olive oil-fed mice was higher than the soybean oil-fed mice at days 5 and 8 after infection (Table 2). In the olive oil-fed mice, isolation stress resulted in a higher liver weight at day 8 post-infection than in the group-housed mice. In the soybean oil-fed mice, by contrast, isolation stress did not significantly affect liver weight. In all groups, the spleen weight increased from 100–113 mg (day 0, before infection with fungus) to 241–270 mg by day 5 after infection, and stayed at elevated levels until day 8. Neither the isolation stress conditions nor the test diets had any significant effect on the spleen weight in these animals (data not shown).

Effects of dietary oils and isolation stress on antifungal resistance

The liver CFU count of the olive oil-fed mice was greater than the soybean oil-fed mice at days 3, 5, and 8

Table 2. Effects of Dietary Oils and Isolation Stress on Liver Weights of *P. brasiliensis*-Infected Mice[1]

Time after infection (d)	Liver weight (g)				Two-way ANOVA[3]
	Olive oil		Soybean oil		
	Group-housed[2]	Isolated	Group-housed	Isolated	
0[†]	1.25 ± 0.11	1.26 ± 0.10	1.21 ± 0.10	1.22 ± 0.05	—
3	1.54 ± 0.23	1.63 ± 0.11	1.57 ± 0.07	1.55 ± 0.10	—
5	1.71 ± 0.09	1.83 ± 0.08	1.52 ± 0.08	1.66 ± 0.11	Oil, House
8	1.57 ± 0.05^b	1.80 ± 0.13^a	1.57 ± 0.14^b	1.53 ± 0.10^b	Oil, Interaction

[†]Day 0, before infection with fungus.
(−) Not significant ($p > 0.05$).
Values in a row with no common superscript letter are significantly different ($p < 0.05$).
[1]Results are expressed as mean ± S.D. (n = 5).
[2]Housing conditions: Group-housed, four mice per cage; Isolated, one mouse per cage.
[3]Significant effect ($p < 0.05$) of test oil (Oil) and housing conditions (House), and significant effect of interaction between test oil and housing conditions (Oil × House).

Table 3. Effects of Dietary Oils and Isolation Stress on Recovery of *P. brasiliensis* in Livers of Mice[1]

Time after infection (d)	Colony forming units				Two-way ANOVA[3]
	Olive oil		Soybean oil		
	Group-housed[2]	Isolated	Group-housed	Isolated	
0[†]	27,612 ± 2,968	29,568 ± 3,653	25,665 ± 3,560	27,915 ± 3,494	—
3	79,407 ± 8,766[b]	101,789 ± 9,527[a]	59,546 ± 5,963[c]	68,496 ± 8,563[bc]	Oil, House, Interaction
5	39,500 ± 5,818	56,216 ± 12,431	35,065 ± 10,685	39,503 ± 6,454	Oil, House
8	12,352 ± 3,160	15,712 ± 7,222	8,432 ± 3,763	8,258 ± 1,877	Oil

[†]Day 0, 3 h after infection with fungus.
(−) Not significant ($p > 0.05$).
Values in a row with no common superscript letter are significantly different ($p < 0.05$).
[1]Results are expressed as mean ± S.D. (n = 5).
[2]Housing conditions: Group-housed, four mice per cage; Isolated, one mouse per cage.
[3]Significant effect ($p < 0.05$) of test oil (Oil) and housing conditions (House), and significant effect of interaction between test oil and housing conditions (Oil × House).

Table 4. Effect of Dietary Oils and Isolation Stress on Liver Levels of IFN-γ in *P. brasiliensis*-Infected Mice[1]

Time after infection (d)	IFN-γ (ng/whole liver)				Two-way ANOVA[3]
	Olive oil		Soybean oil		
	Group-housed[2]	Isolated	Group-housed	Isolated	
0[†]	16.4 ± 2.9	19.3 ± 4.7	17.3 ± 3.2	18.1 ± 2.8	—
3	36.4 ± 5.7	33.0 ± 4.4	35.6 ± 3.3	31.6 ± 4.1	—
5	37.8 ± 6.6	46.4 ± 2.1	29.1 ± 3.7	36.2 ± 2.7	Oil, House
8	18.1 ± 2.7[b]	24.2 ± 3.6[a]	17.5 ± 3.0[b]	16.9 ± 3.2[b]	Oil, Interaction

[†]Day 0, before infection with fungus.
(−) Not significant ($p > 0.05$).
Values in a row with no common superscript letter are significantly different ($p < 0.05$).
[1]Results are expressed as mean ± S.D. (n = 5).
[2]Housing conditions: Group-housed, four mice per cage; Isolated, one mouse per cage.
[3]Significant effect ($p < 0.05$) of test oil (Oil) and housing conditions (House), and significant effect of interaction between test oil and housing conditions (Oil × House).

after infection (Table 3). In the olive oil-fed mice, isolation stress resulted in a greater CFU count in the liver at day 3 post-infection than in the group-housed animals. In contrast, in the soybean oil-fed mice, isolation stress was observed not to significantly affect the liver CFU count. In all groups, spleen CFU counts progressively decreased from 6,110–6,865 CFU/whole organ (3 h after infection) to 704–1,074 CFU/whole organ by day 8 after infection. At day 5 post-infection, the spleen CFU count was greater in the isolation-stressed mice (4,486 ± 838 CFU/whole organ, n = 10) than the group-housed animals (3,414 ± 833 CFU/whole organ, n = 10), but was not affected by dietary oils (data not shown).

Effects of dietary oils and isolation stress on infection-induced cytokine production

Since IFN-γ is a major mediator of host resistance against *P. brasiliensis* infection,[14] the effects of dietary oils and isolation stress on the infection-induced IFN-γ response was investigated. The olive oil-fed mice showed greater IFN-γ production in the liver at days 5 and 8 post-infection than the soybean oil-fed mice (Table 4). At day 5 after infection, liver IFN-γ production was greater under conditions of isolation stress than under group-housing conditions. In the olive oil-fed mice, isolation stress resulted in greater IFN-γ production at day 8 after infection than in the group-housed animals. In contrast, in the soybean oil-fed animals, isolation stress had no significant effect on the liver IFN-γ production at day 8. As shown in Table 5, greater infection-induced IL-6 production in the liver was observed in the olive oil-fed mice at days 5 and 8 post-infection than in the soybean oil-fed mice.

In the spleen, IFN-γ and IL-6 constitutively existed (IFN-γ, 0.55–0.64 ng/whole organ; IL-6, 69–90 pg/whole organ). In all groups, the spleen IFN-γ and IL-6 levels increased to 2.52–3.95 ng/whole organ and 134–167 pg/whole organ by day 5 post-infection respectively. At day 3 after infection, spleen IFN-γ production was greater in the isolation-stressed mice (3.04 ± 0.45 ng/whole organ, n = 10) than in the group-housed animals (2.71 ± 0.42 ng/whole organ, n = 10). Spleen IL-6 production was not affected by dietary oils or isolation stress conditions (data not shown).

Discussion

In the present study, in olive oil-fed but not soybean oil-fed mice, isolation stress results in a temporarily delayed clearance of *P. brasiliensis* in the liver as compared with group-housed counterparts. This indicates that soybean oil rich in n-6 polyunsaturated fatty acids is more beneficial than olive oil high in monounsaturated fatty acids in attenuating isolation stress-induced modulation of host defense against *P. brasiliensis*. It has been reported that decreases in AA content of immunocompetent cells are accompanied by suppressed production of proinflammatory cytokines in these cells.[7,8] In our previous study, isolation stress resulted in a reduced AA content of lymphocytes, markedly and moderately in the olive oil-fed and

Table 5. Effects of Dietary Oils and Isolation Stress on Liver Levels of IL-6 in *P. brasiliensis*-Infected Mice[1]

Time after infection (d)	IL-6 (ng/whole liver)				Two-way ANOVA[3]
	Olive oil		Soybean oil		
	Group-housed[2]	Isolated	Group-housed	Isolated	
0[†]	3.00 ± 0.86	2.74 ± 0.06	2.28 ± 0.42	2.53 ± 0.30	—
3	4.01 ± 0.68	3.26 ± 0.71	3.42 ± 0.36	3.01 ± 0.48	—
5	5.07 ± 0.78	5.44 ± 0.31	4.00 ± 0.47	3.84 ± 0.21	Oil
8	2.98 ± 0.18^{ab}	3.22 ± 0.34^{a}	2.95 ± 0.56^{ab}	2.35 ± 0.31^{b}	Oil, Interaction

[†]Day 0, before infection with fungus.
(−) Not significant ($p > 0.05$).
Values in a row with no common superscript letter are significantly different ($p < 0.05$).
[1]Results are expressed as mean ± S.D. (n = 5).
[2]Housing conditions: Group-housed, four mice per cage; Isolated, one mouse per cage.
[3]Significant effect ($p < 0.05$) of test oil (Oil) and housing conditions (House), and significant effect of interaction between test oil and housing conditions (Oil × House).

soybean oil-fed mice respectively.[4] In addition, isolation stress led to decreased IFN-γ production in concanavalin A-stimulated lymphocytes from olive oil-fed but not soybean oil-fed mice, and caused reduced IL-6 production in these lymphocytes from olive oil- and soybean oil-fed mice.[4] In contrast, in the present study, in the olive oil-fed mice, isolation stress enhanced rather than suppressed infection-induced IFN-γ production in the liver as compared with group-housed counterparts (Table 4). Furthermore, in the olive oil- and soybean oil-fed mice, isolation stress did not reduce infection-induced liver IL-6 production as compared with the group-housed animals (Table 5). The mechanisms underlying these opposite effects of social isolation stress on IFN-γ and IL-6 productions should be further investigated.

IFN-γ and proinflammatory cytokines play a critical role in host resistance to infection.[15,16] Treatment of mice with anti-IFN-γ monoclonal antibody resulted in delayed clearance of *Bordetella pertussis* in the respiratory tract.[17] In our study, the olive oil-fed mice subjected to isolation stress showed a temporarily delayed clearance of the fungus in the liver at day 3 after infection (Table 3) and greater liver IFN-γ production at day 8 (Table 4) than the other three groups. In addition, infection-induced liver IL-6 production was also greater in the olive oil-fed mice subjected to isolation stress at day 8 post-infection than in the soybean oil-fed mice subjected to that stress (Table 5). Cao and Lawrence[18] have reported that acute cold/restraint stress-treated mice exhibit delayed clearance of bacteria from the spleen and liver, and greater production of IFN-γ and pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in these organs following *Listeria monocytogenes* infection as compared with unstressed mice. Avitsur *et al.*[12] have reported that adult mice that as pups experienced handling with maternal separation show delayed clearance of influenza virus from the lung and greater upregulation of IL-6, IL-12, and IFN-γ mRNA levels in this organ than unstressed mice. The greater response of IFN-γ and proinflammatory cytokines might result from the increased magnitude of infectious burden, as suggested by Ashman *et al.*,[19] who observed that *Candida albicans* infection increased the level of IFN-γ mRNA in the mouse brain in proportion to the magnitude of the infectious burden, and that the increased response of IFN-γ then contributed to the clearance of the yeasts from the brain. On the other hand, it has been reported that overproduction of IFN-γ is also responsible for the increased lethality observed in mice infected with *Candida albicans*[20] and *Toxoplasma gondii*,[21] and that treatment with an anti-IFN-γ antibody improves the survival of mice infected with gram-negative bacteria.[22] It has been proposed that IL-6, although critical to establishing antimicrobial defenses, contributes to pathogenesis when released in excess.[23] Hence, it is presumable that the greater liver IFN-γ and IL-6 responses in the olive oil-fed mice subjected to isolation stress is more detrimental to the host than to the other groups, although it might simultaneously contribute to host resistance against *P. brasiliensis*. This is the first indication that psychological stress-induced changes in host resistance to infection vary depending on dietary fatty acid composition. The reason for the different effects of dietary olive oil and soybean oil on psychological stress-induced modulation in host resistance remains to be elucidated.

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