

Short communication

Cold-pressed flaxseed oil reverses age-associated depression in a primary cell-mediated adaptive immune response in the mouse

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The objective of this investigation was to determine the influence of flaxseed oil on responses representative of primary humoral and cell-mediated adaptive immune competence in immunosenescent mice. Male and female C57BL/6J mice, 85 weeks old, were randomized between two complete purified diets differing only in oil source (cold-pressed safflower or flaxseed). After 8 weeks, humoral competence was assessed in six mice per group as the serum haemagglutinin titre to sheep red blood cells (SRBC) and cell-mediated competence was assessed, in an additional six mice per group, as the delayed hypersensitivity response to SRBC. A zero-time control group (88 weeks old) and a young adult positive control group (12 weeks old) were each tested similarly (six per immune response), revealing age-related depression in both antibody and cell-mediated competence at 88 weeks of age. After the 8-week experimental period, the antibody response of the two test groups of geriatric mice remained below the young adult level ($P=0.04$) and the cell-mediated response of the safflower oil group also continued to exhibit age-related depression (20% of young adult level, $P=0.0002$). By contrast, the anti-SRBC delayed hypersensitivity response of the flaxseed group no longer differed from the response of the young adults but exceeded that of the safflower and zero-time control senescent groups ($P=0.0002$). Depression in primary cell-mediated competence, the most outstanding aspect of immunosenescence, can be addressed by means of a dietary source of 18:3n-3 without longer-chain PUFA.

Ageing: Cell-mediated immunity: Flaxseed oil: Mouse

Depression in thymus-dependent immune competence is considered an important contributor to the high risk of infection among the elderly (Ginaldi *et al.* 2001; Bogden & Louria, 2004). In turn, prostaglandin E₂ (PGE₂) is implicated as a mediator of immunosenescence (Beharka *et al.* 1997). Therefore, strategies that reduce the capacity to produce this eicosanoid could be expected to promote adaptive immune competence in the latter stages of life. Dietary replacement of n-6 fatty acids with n-3 acids, either from fish oil or from flaxseed oil, reduces the capacity for PGE₂ production (Caughey *et al.* 1996). Nevertheless, fish oil supplements depressed cellular immune competence, albeit modestly, in elderly human subjects (Meydani *et al.* 1991; Bechoua *et al.* 2003) and geriatric animals (Wander *et al.* 1997). As discussed by Fan *et al.* (2003), fish oil is generally depressive of thymus-dependent lymphocyte functions, an outcome substantially attributable to non-eicosanoid-mediated actions of 20:5n-3 and 22:6n-3. Flaxseed oil provides n-3 fatty acids exclusively as 18:3n-3 which is inefficiently elongated and desaturated (Holub & Holub, 2004). Therefore, consumption of flaxseed oil might minimize the non-eicosanoid-mediated depressive influences on immune competence associated

with high-level intake of long-chain n-3 polyunsaturates while permitting sufficient production of 20:5n-3 to counteract eicosanoid-mediated immune senescence. The objective of this investigation was to determine the influence of flaxseed oil on responses representative of primary thymus-dependent competence in immunosenescent mice.

Materials and methods

Animals, diets and animal facility

Male and female C57BL/6J mice were used from an in-house breeding colony. Prior to the experiment, the mice were caged in groups of up to four animals, males and females separately, and were fed a commercial rodent chow (Purina Rodent Chow #5001, Ralston, Purina Mississauga, Ontario, Canada). Purified diets for the investigation differed only in oil source, i.e. cold-pressed flaxseed or safflower oils (Omegaflo[®]), provided as a gift from Omega Nutrition Canada, Inc. (Vancouver, BC, Canada). These diets are described elsewhere (Hillyer & Woodward, 2002) and contained 80 g of their respective oil source per kg. While

on experiment, the mice were caged individually, had free access to diet and tap water, and were permitted coprophagy. The animals were maintained from birth, and throughout the experiment, in the same windowless room maintained at 25–27°C and 60% relative humidity with a photoperiod of 14 h fluorescent light and 10 h darkness. Animal care and experimental usage were conducted according to the guidelines of the Canadian Council on Animal Care and all aspects of this work were approved by the University of Guelph Animal Care Committee.

Experimental design

At a mean age of 85 weeks (range 78–104 weeks) males and females were separately randomized between the two purified diets (i.e. containing either safflower or flaxseed oil) for an 8-week feeding period. Each dietary group included eight males and eight females. At the end of the experimental period, humoral and cell-mediated competence were each assessed in three males and three females of each dietary group. In addition, two chow-fed control groups were included, namely a zero-time control group (mean 88 weeks of age; range 83–99 weeks) consisting of seven males and ten females and a young adult positive control group (mean 12 weeks of age; range 11.5–13 weeks) consisting of eight males and nine females. Humoral competence was assessed in two males and four females from the zero-time control group and in three males and three females from the positive control group. Cell-mediated competence was assessed in three males and four females from each of the zero-time and positive control groups. Two animals (one male and one female) from each dietary and control group served as unsensitized negative controls for each immune response.

Blood sampling and serum storage

A blood sample from the orbital plexus was taken from each test mouse at the end of the experimental period (day 56) and also from each mouse of the two control groups. The procedure including storage of serum samples is described elsewhere (Hillyer & Woodward, 2002).

Chemical analyses of serum, dietary oils and carcasses

Fatty acid analyses of serum and dietary oils were performed by capillary GC as described elsewhere (Hillyer & Woodward, 2002). Likewise, the procedures used to determine the DM and lipid contents of the animal carcasses are outlined elsewhere (Hillyer & Woodward, 2002).

Assessment of humoral immune competence

Humoral competence was assessed as the primary antibody response to the sheep red blood cell (SRBC; CedarLane, Hornby, ON, Canada) according to a published procedure (Hillyer & Woodward, 2002). The immunizing dose of SRBC, or the negative control injection of saline carrier, was given to mice of the test groups on day 51 of the experimental period and serum agglutinin titre (i.e. concentration of anti-SRBC antibody) was determined on day 56. Titre was determined as the inverse base 2 logarithm of the maximum

serum dilution at which agglutination of SRBC was discernible with the unaided eye and was discerned by an observer (B. W.) blinded to the dietary code. In this system each difference of one integer in titre (e.g. 7.5 v. 8.5) reflects a two-fold difference in anti-SRBC antibody concentration.

Assessment of cell-mediated immune competence

Cell-mediated competence was assessed as the primary delayed hypersensitivity response to SRBC as described elsewhere (Ha *et al.* 2001). An immunizing dose of SRBC, or a negative control injection of saline carrier, was given to mice of the test groups on day 51 of the experimental period. Footpad challenge with SRBC was performed on day 55 and the resulting local inflammatory response was quantified by a blinded observer (B. W.) 24 h later as the quotient of the maximum thickness of the SRBC-challenged (right) foot divided by the maximum thickness of the saline-challenged (left) foot. The response of unsensitized negative control mice was subtracted from the response of sensitized animals to yield the estimate of adaptive (i.e. lymphocyte-mediated) immune competence.

Statistical analyses

Statistical analysis was performed according to the Statistical Analysis Systems software package version 8 (SAS Institute, Cary, NC, USA). The predetermined upper limit of probability for statistical significance throughout this investigation was $P \leq 0.05$, and means comparisons were two-tailed. Data sets were either subjected to two-way ANOVA (to permit inclusion of gender as a main effect) or, if not normally distributed according to each of the four tests applied by the SAS program, were analysed using the Kruskal–Wallis test (χ^2 approximation) which was applied to Wilcoxon rank sums. *Post hoc* testing, where justified by the statistical probability (i.e. $P \leq 0.05$), was done using either Tukey's studentized range procedure or χ^2 comparisons of Wilcoxon two-sample rank sums, as appropriate.

Results

Growth indices and serum fatty acid profiles are shown in Table 1. Gender-related effects were not remarkable and are not shown. Initial mean body weights of the two test groups and the zero-time control did not differ, but exceeded the mean body weight of the younger adult positive control group ($P=0.0001$) as a reflection of fat accumulation by the older animals ($P=0.0001$) which increased their mean carcass DM content above that of the young adult group ($P=0.0004$). Food intake and final body weight were not affected by dietary oil source ($P=0.57$ and $P=0.29$, respectively), and weight change was unapparent during the 8-week experimental period (Student's *t* test, $P=0.19$). The fatty acid composition expected of the oil sources was confirmed by chemical analysis (results not shown) and was similar to results reported previously (Hillyer & Woodward, 2002). In turn, the serum *n-3* and *n-6* fatty acid profiles of the test groups reflected the composition of their respective oil sources. Serum total fatty acid concentration was not affected by dietary oil source (results not shown).

Table 1. Growth indices and serum fatty acid profiles*

Index	Group of mice												Pooled SEM
	Positive control†			Zero-time control‡			Safflower oil§			Flaxseed oil§			
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	
Body weight (g/mouse)													
Initial	22.2 ^b			41.5 ^a			44.0 ^a			41.0 ^a			1.11
Final	–			–			41.3			39.0			1.47
Food intake (g/mouse per d)	–			–			5.2			5.3			0.13
Carcass (g/kg wet wt)													
DM	32.5 ^b			42.9 ^a			45.3 ^a			47.5 ^a			2.51
Lipid	8.8 ^b			22.4 ^a			24.1 ^a			26.4 ^a			2.22
Serum fatty acid levels (µg/ml)													
Total <i>n</i> -6		105 ^{ab}	51–249		99 ^b	31–143		113 ^a	43–272		39 ^c	6–103	–
20:4 <i>n</i> -6¶		25 ^b	13–65		20 ^b	12–68		54 ^a	15–165		9 ^c	0–32	–
Total <i>n</i> -3**		20 ^b	0–77		11 ^b	0–151		10 ^b	0–81		66 ^a	20–216	–
20:5 <i>n</i> -3††		0.4 ^b	0–6		0.3 ^b	0–4		0.5 ^b	0–6		18 ^a	4–92	–

^{a,b,c} Values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* Mean values are shown where parametric statistics were applied (indicated by pooled SEM; either two-tailed Student's *t* test or two-way ANOVA followed by Tukey's studentized range test). Median values (range) are shown where data sets were subjected to the Kruskal–Wallis test followed by comparisons of Wilcoxon two-sample rank sums. Outcomes pertain to diet main effect only. For details of diets and procedures, see p. 230.

† Young adult positive control group, mean age 12 weeks.

‡ Zero-time control group, mean age 88 weeks.

§ Test groups fed purified diets formulated with either safflower or flaxseed oils for 8 weeks, mean initial age 85 weeks.

|| Kruskal–Wallis test of Wilcoxon rank sums ($P=0.0003$) which were 525.0 (positive control), 351.5 (zero-time control), 487.5 (safflower) and 171.0 (flaxseed).

¶ Kruskal–Wallis test of Wilcoxon rank sums ($P=0.0009$) which were 462.0 (positive control), 366.5 (zero-time control), 516.5 (safflower) and 195.0 (flaxseed).

** Kruskal–Wallis test of Wilcoxon rank sums ($P=0.0031$) which were 446.5 (positive control), 295.0 (zero-time control), 268.5 (safflower) and 530.0 (flaxseed).

†† Kruskal–Wallis test of Wilcoxon rank sums ($P=0.0001$) which were 323.0 (positive control), 302.0 (zero-time control), 284.0 (safflower) and 631.0 (flaxseed).

The assessments of primary immune competence are shown in Fig. 1 independently of gender-related effects which did not affect the interpretation of results and are not shown. Anti-SRBC titres did not differ among the three groups of older mice, but the range of titres exhibited by these groups fell below the range of antibody concentrations detected in the serum of the young adult positive control group ($P=0.04$). This manifestation of immunosenescence could reflect either a delay in response kinetics or a decline in peak titre, or both. In this laboratory, the primary anti-SRBC antibody titre peaks between 4 and 6 d after sensitization in mice ranging from 3 weeks of age (early weaned) to immunosenescence at approximately 70 weeks (results not shown). Unsensitized mice failed to exhibit a detectable serum anti-SRBC titre. By contrast with the antibody response, both age and oil source affected the cell-mediated response ($P=0.0002$). The mean delayed hypersensitivity response of the zero-time control group was approximately 40% of the response of the young adult group. Whereas the safflower group did not differ from the zero-time control in this respect, the mean cell-mediated response of the flaxseed oil group exceeded that of the safflower and zero-time control groups, but did not differ from the mean response generated by the young adult animals. This data set provided a statistical power of 90% for detection of a 50% difference between means *vis-à-vis* the main effect of oil source and age group. The anti-SRBC inflammatory response of negative control (unsensitized) mice did not differ among groups and so a collective mean of the responses exhibited by the unsensitized mice, 2.6 (SD 1.2) %, was calculated across the four groups of animals and was applied to all groups to produce the results shown in Fig. 1. Finally, females generated a larger delayed

hypersensitivity response than males (19% *v.* 15%, $P=0.04$), but the influence of oil source and age group on the cell-mediated response was independent of gender (interaction term $P=0.42$).

Discussion

In the present investigation, dietary intervention with an *n*-3-rich, *n*-6-poor plant oil effected reversal of age-associated depression in a primary cell-mediated immune response. In broad perspective, the results are consistent with the viewpoint (Bogden & Louria, 2004) that immunosenescence may substantially reflect external influences rather than intrinsic change. This outcome was independent of gender and was achieved in a cohort of demonstrably immunosenescent animals. Similar immunological rejuvenation has been reported in elderly human subjects consequent to micronutrient interventions (Bogden & Louria, 2004), although formal evidence of a return to a young adult level of cell-mediated competence has not been presented previously. Moreover, an outstanding characteristic of immunosenescence is a particular decline in the capacity to generate primary responses (Ginaldi *et al.* 2001), whereas previous evidence relating to stimulation of cell-mediated competence in advanced age (Bogden & Louria, 2004) has pertained either to recall responses or to the surrogate marker of mitogen-induced blastogenesis.

The cold-pressed flaxseed oil used in this investigation supported more rapid ontogeny of adaptive immune competence in the weanling mouse than *n*-6-rich oils (Hillyer & Woodward, 2002) but provided comparable support of immune responses in young adult mice (K. Newell, unpublished results). Therefore, flaxseed oil emerges in particular support

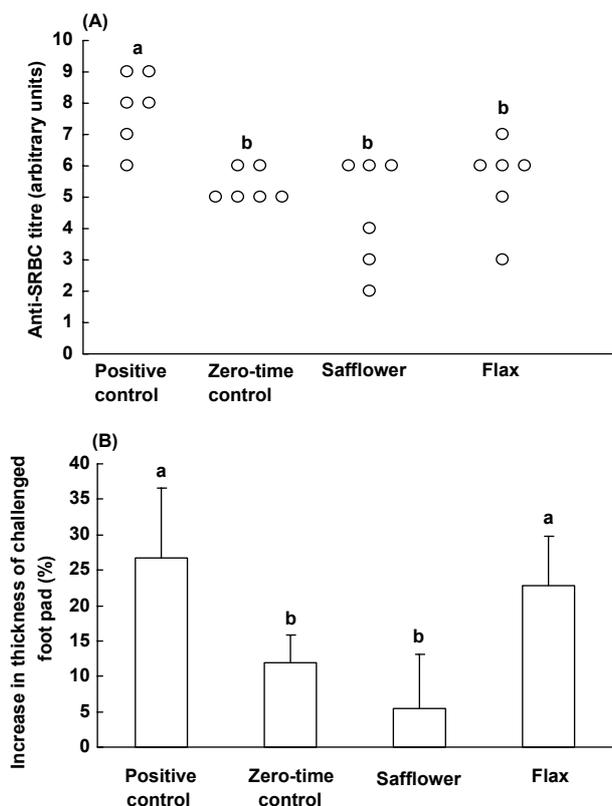


Fig. 1. Primary antibody and cell-mediated responses to sheep red blood cell (SRBC) by 85-week-old experimental, 12-week-old positive control and 88-week-old zero-time control groups of male and female mice. Experimental animals were fed, for 8 weeks, purified diets formulated to contain 80 g cold-pressed safflower or flaxseed oil per kg. Each mouse was sensitized with SRBC so as to elicit either a cellular or a humoral response. For details of diets and procedures, see p. 230. (A), Anti-SRBC antibody titres. Each point represents one mouse, and groups with unlike superscript letters were significantly different ($P \leq 0.05$). Kruskal–Wallis procedure (diet: $P = 0.0369$; Wilcoxon rank sums: 118.0, 61.0, 62.5 and 58.5 for the positive control, zero-time control, flaxseed and safflower groups, respectively) followed by χ^2 comparisons of Wilcoxon two-sample rank sums. (B), Anti-SRBC delayed hypersensitivity responses. Values are means with their standard deviations depicted by vertical bars. Mean values with unlike superscript letters were significantly different ($P \leq 0.05$). Two-way ANOVA (diet: $P = 0.0002$; gender: $P = 0.0377$; interaction: $P = 0.416$ (pooled SEM 2.01)).

of immune competence during the physiologically labile early and late stages of life. This may reflect, primarily, a reduced intake of $n-6$ fatty acids (Hillyer & Woodward, 2002), and an $n-3$ - and $n-6$ -poor oil source such as olive oil deserves attention in this respect.

The serum levels of 20:4 $n-6$ and 20:5 $n-3$ in the mice fed flaxseed oil suggest reduced production of PGE₂ relative to the animals consuming safflower oil and also relative to the two control groups. This would be expected to promote cell-mediated competence while exerting a less predictable influence on antibody responses (Betz & Fox, 1991) and provides some basis for speculating that an eicosanoid-mediated mechanism predominates in the action of flaxseed oil reported herein. This is the primary means, for example, whereby megadoses of vitamin E are thought to promote immune competence at advanced age (Beharka *et al.* 1997).

Cell-mediated responses defend against intracellular infectious agents (Ginaldi *et al.* 2001). Flaxseed oil, therefore, may improve resistance to viruses and facultative intracellular

parasites in the late stages of life, an important possibility in view of the viral and tuberculous respiratory infections common to the elderly (Ginaldi *et al.* 2001). On the other hand, autoimmune disease is a well-recognized age-related phenomenon (Ginaldi *et al.* 2001; Bogden & Louria, 2004), and immunosenescence may reduce this risk. Direct evidence of rejuvenation of primary cell-mediated competence in advanced age is truly noteworthy, but it would be premature to conclude that this will prove exclusively beneficial.

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