

The ratio of n-6 to n-3 fatty acids in maternal diet influences the induction of neonatal immunological tolerance to ovalbumin

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SUMMARY

Prevalence of allergy is increasing in many countries and might be related to changed environmental factors, such as dietary fatty acids (FA). The present study investigates whether dietary ratio of n-6 to n-3 FA influences the induction of immunological tolerance to ovalbumin (OA) in neonatal rats. During late gestation and throughout lactation Sprague-Dawley rats were fed a diet containing 7% linseed oil (n-3 diet), sunflower oil (n-6 diet) or soybean oil (n-6/n-3 diet). At 10–16 days of age the rat offspring were subsequently exposed, or not, to OA via the milk. The offspring were weaned onto the same diets as the mothers and immunized with OA and the bystander antigen human serum albumin (HSA). In the offspring on the n-3 diet exposure to OA via the milk resulted in lower delayed type hypersensitivity reaction (DTH) and antibody responses against both OA and HSA, compared to those in the offspring not exposed to OA, indicating the induction of oral tolerance. In the offspring on the n-6 diet, the exposure to OA led to depressed specific immune responses against only OA, not HSA. In the offspring on the n-6/n-3 diet oral exposure to OA did not influence immune responses against OA, or HSA. The results indicate that the dietary ratio of n-6/n-3 FA is important for the induction of neonatal oral tolerance. Thus nonoptimal feeding may have effects on the development of immunological tolerance to dietary antigen ingested by the mother. The ratio of n-6/n-3 FA in the diet may be considered in the context of increased prevalence of allergy.

Keywords oral tolerance diet fatty acids lactation neonatal period

INTRODUCTION

Prevalence of allergy is increasing in many countries and might be related to changed environmental factors, such as the dietary fatty acid (FA) pattern [1–3]. Over the last 50 years the consumption of saturated fat and n-6 FA has been substantially increased, while the consumption of n-3 FA derived from plant and marine food has declined, resulting in increased ratios of n-6/n-3 FA in many countries towards 10:1–16:1 [4,5]. Epidemiological studies suggest that the increased intake of n-6 FA in the Western diet might be one triggering factor for the increased prevalence of atopic diseases [1–3], including allergic sensitization to food antigens. The levels of n-6 and n-3 FA in the breast milk are determined to a large extent by the maternal diet and vary significantly depending on dietary habits and geographical location [6]. Recent studies on the protective role of breastfeeding against allergic diseases have shown contradictory results [7,8], but these studies have not analysed the lipid composition of the milk. The variations of FA

composition of human milk might in part explain some of the controversies. Indeed, lower levels of n-3 FA, as well as disturbed relationships between the n-3 FA and the n-6 FA in mature milk from the mothers, appear to be associated with atopic sensitization early in life [9].

Usually, oral exposure to food antigens results in an induction of oral tolerance, a state of specific immunological hyporesponsiveness upon further exposure to antigen [10–12]. Failure to develop immunological tolerance leads to an immune response resulting in allergic sensitization to food antigens [13]. Factors important for the induction or breakdown of oral tolerance in the neonatal period are poorly understood. One of these factors might be the balance between n-6 and n-3 FA in the diet. Dietary n-6 and n-3 FA influence the membrane structure of immune cells, eicosanoid and cytokine production, immunoglobulin and adhesion molecule synthesis and affect immune responsiveness to antigens [14,15]. Dietary intake of polyunsaturated FA (PUFA) has been shown to influence the tolerance induction in adult mice [16] and the development of immunological tolerance to dietary antigen in rat offspring [17]. Furthermore, different effects of dietary n-6 and n-3 FA on Th1 and Th2-like responses and the induction of oral tolerance to ovalbumin (OA) have been

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demonstrated in adult mice [18]. However, whether the ratios of n-6 and n-3 FA in the maternal diet influence the induction of neonatal immunological tolerance in the offspring has not been studied previously.

The aim of the present study was to investigate the effects of different ratios of n-6 to n-3 FA in the maternal diet on the induction of the neonatal oral tolerance in the rat offspring.

MATERIALS AND METHODS

Animals and diets

Pregnant Sprague-Dawley rats (BK Universal, Stockholm, Sweden) were received on day 7 of gestation and kept in our animal facility under constant conditions of humidity (70–80%), temperature (22–25°C), and light (12-h light and dark cycle). The rats were housed individually in plastic cages with food and water available *ad libitum*. Ten days before delivery the rats were assigned to one of three groups ($n = 9$ –10 in each group) receiving different diets (Morinaga Milk Industry Co. Ltd, Tokyo, Japan). The diets differed only by lipid composition: 7% linseed oil (n-3 diet), soybean oil (n-6/n-3 diet), or sunflower oil (n-6 diet) with the ratios of n-6 to n-3 EFA of 0.4, 9 and 216, respectively. The composition of the three diets is given in Table 1. Litter size was adjusted to 10 pups per litter. The litters were kept with their mothers until day 21 and after weaning received the same diet as the mothers. The pups from the same litters were used for other studies, which will be reported elsewhere.

Table 1. Composition of the diets

Diet	n-3 diet (%)	n-6/n-3 diet (%)	n-6 diet (%)
Casein	20.0	20.0	20.0
Potato starch	54.0	54.0	54.0
Glucose	10.0	10.0	10.0
Cellulosal flour	4.0	4.0	4.0
Mineral mix*	4.0	4.0	4.0
Vitamin mix†	1.0	1.0	1.0
Linseed oil	7.0		
Soybean oil		7.0	
Sunflower oil			7.0
Fatty acids (mol%)			
16:0	14	12	8
16:1	0.3	0.1	0.1
18:0	6.1	3.9	4.2
18:1	32	21	22
18:2(n-6)	14	56	65
20:0	0.3	0.4	0.3
18:3(n-3)	33	6.2	0.3
n-6/n-3	0.4	9	216

*Salt mixture containing (wt%): KH_2PO_4 (34.1); CaCO_3 (35.9); KCl (2.5); NaCl (18); $\text{MgSO}_4 \cdot \text{xH}_2\text{O}$ (5.1); $\text{FeC}_6\text{H}_5\text{O}_7 \cdot \text{xH}_2\text{O}$ (3.3); MnO (0.27); $\text{Cu}_2\text{C}_6\text{H}_4\text{O}_7 \cdot \text{xH}_2\text{O}$ (0.06); ZnCO_3 (0.04); $\text{CoCl}_2 \cdot \text{xH}_2\text{O}$ (0.002); $\text{KAl}(\text{SO}_4)_2 \cdot \text{xH}_2\text{O}$ (0.008); NaF (0.025); KIO_3 (0.009); $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{xH}_2\text{O}$ (0.002); $\text{Na}_2\text{SeO}_3 \cdot \text{xH}_2\text{O}$ (0.001); $\text{Na}_2\text{MoO}_4 \cdot \text{xH}_2\text{O}$ (0.001). †Vitamin mixture containing: Vit A 11.9 IU/g. Vit D₃ 1.5 IU/g. Vit B₁ 4 µg/g. Vit B₂ 12 µg/g. Vit B₆ 5 µg/g. Ca-pantotenate 45% 11 µg/g. Niacin 40 µg/g. Vit B₁₂ 0.02 µg/g. Vit K₃ 7.75 µg/g. Biotin 2% 3 µg/g. Vit C 500 µg/g. Inositol 30 µg/g. Vit E 42 µg/g. Choline chloride 50% 1 mg/g. Folic acid 0.5 µg/g.

The study was approved by the Animal Ethic's Committee of Göteborg University.

Ovalbumin (OA) administration to the dams and immunization of the neonatal rats

In order to induce oral tolerance against OA suckling neonatal rats were exposed to the antigen via breast milk after administration of OA to lactating mothers, which have been shown to induce oral tolerance in the offspring [19]. The dams of each dietary group were given either drinking water with OA (grade V, Sigma Chemical Co., St.Louis, MO, USA) (200 mg/per day and rat) or just water as control on days 10–16 of lactation (Fig. 1). At 3 weeks of age pups randomised from each litter ($n = 9$ –10/group) were immunized s.c in the legs with 100 µg OA and 100 µg human serum albumin (HSA) (grade V, Sigma) in complete Freund's adjuvant. Delayed-type hypersensitivity (DTH) reactions were tested 2 weeks after the immunization. Three weeks after the primary immunization the rats were boosted s.c. with 100 µg OA and 100 µg HSA in incomplete Freund's adjuvant. One week after the booster dose the animals were killed using CO_2 .

Blood samples for antibody determination were taken from the tip of the tail of the rats. The draining popliteal lymph nodes (PLN) were removed and weighed. Truncal blood was collected at 3 weeks of age and sera were kept frozen (−20°C) until analyses of FA composition of serum phospholipids (PL).

Fatty acid analysis

Total lipids of serum were extracted according to Folch *et al.* [20], fractionated on a single SEP-PAK aminopropyl cartridge (Waters Corp., Massachusetts, USA) [21] and the fraction of PL was analysed. The FA methyl esters were separated by capillary gas-liquid chromatography in a Hewlett-Packard 6890 gas chromatograph according to the method described previously [22]. The separation was recorded with HP GC Chem Station software (HP GC, Wilmington, DE, USA). The FA 21:1 was used as internal standard and the FA methyl esters identified by comparison with retention times of pure reference substances (Sigma Aldrich Sweden AB, Stockholm, Sweden).

Antibody determinations

Serum IgG and IgM anti-OA antibody levels were determined by ELISA as follows.

Microtitre plates (MIC-2000, Dynatech, Alexandria, VA, USA) were coated overnight with 5 µg/ml OA or HSA in PBS. The serum samples were diluted in 100 µl PBS-Tween (0.05%), 1/100 for IgG and 1/50 for IgM determinations. Every sample was then serially diluted (1/5 for IgG and 1/3 for IgM for six steps), added to the plates and plates were incubated for 3 h at room temperature. Rabbit anti-rat IgG antibodies, dilution: 1/10 000 (Zymed Laboratories Inc., San Francisco, CA, USA), or rabbit anti-rat IgM antibodies, dilution: 1/20 000 (Jackson Immuno Research Laboratories Inc., West Baltimore Pike) were then added to the plates and incubated overnight at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit-IgG or anti-rabbit-IgM, diluted 1/10 000 (Sigma) were then added to the plates and incubated 1 h. The plates were washed and the reactions were visualized with the substrate p-nitrophenyl phosphate (1 mg/ml, Sigma) dissolved in diethanolamine buffer, pH 9.8. The absorbance was measured with a spectrophotometer at 405 nm (Titertek Multiskan, Flow Laboratories, MacLean, VA, USA) after 60 min. The plates were washed with PBS-Tween between

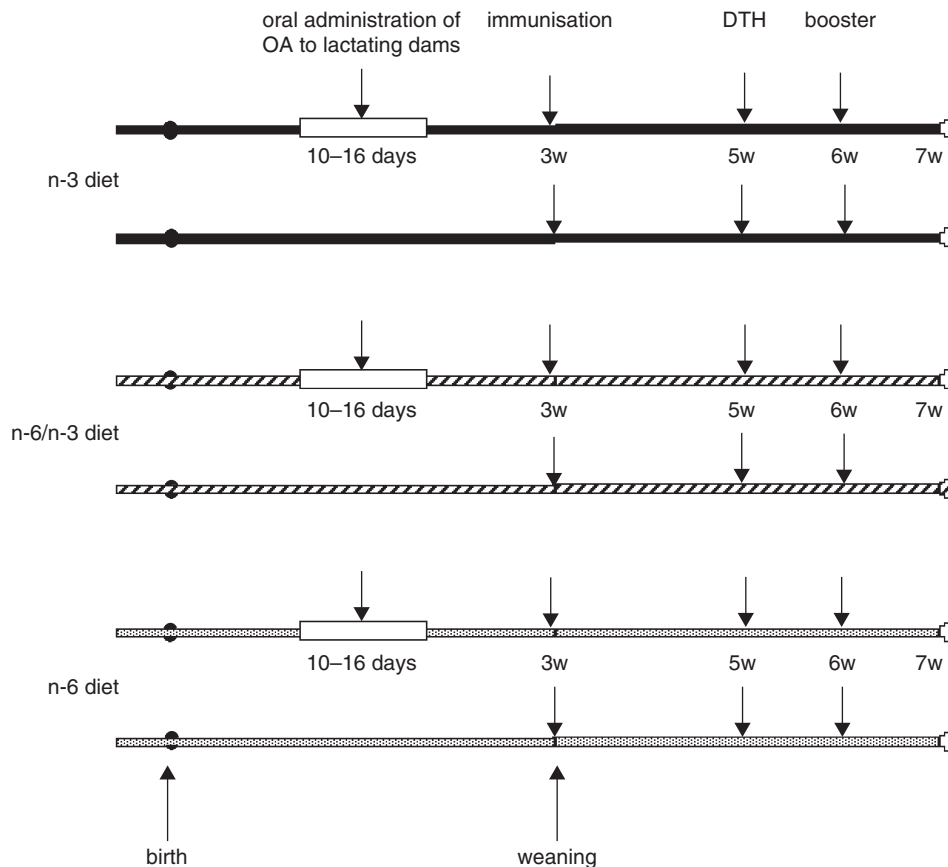


Fig. 1. The protocol for the immunization of neonatal offspring of dams fed the diet with different ratios of n-6 to n-3 fatty acids. During late gestation and throughout lactation rat dams were fed the different diets and the offspring were weaned onto the same diet as the mothers. To expose the rat offspring to OA via the milk the dams of each dietary group were given either drinking water with OA or just water as control on days 10–16 of lactation. At 3 weeks of age the offspring were immunized with OA and HSA. DTH reactions were tested 2 weeks after the immunization. Three weeks after the primary immunization the rats were boosted with OA and HSA. One week after the booster dose the animals were sacrificed.

the incubations. The antibody levels were expressed in arbitrary ELISA units calculated from a standard curve obtained with a pool of hyperimmune serum.

Serum IgE anti-OA antibody levels were determined by the passive cutaneous anaphylaxis reaction (PCA) as follows. Adult male Sprague-Dawley rats were injected intradermally with serum of the immunized animals from each dietary group. The sera were injected s.c. undiluted and diluted (1:2 in five steps) on the back in a volume of 50 μ l. After 72 h 5 mg OA in 1% Evan's blue solution (Sigma) in PBS was injected intravenously in 1 ml volume. Thirty minutes later the rats were killed and the diameter of the reaction measured on the skin. Data were expressed as PCA titres: the highest dilution of a serum sample that gave a spot with more than 5 mm diameter.

Delayed-type hypersensitivity (DTH)

The DTH responses to OA and HSA were tested 2 weeks after the primary immunization by injecting 50 μ g of antigen in 20 μ l of PBS s.c. in the ear. OA and HSA were injected in separate ears. The ear thickness was measured before and 24 h after injection, using a micrometer caliper (Oditest, Kröplin, Hessen, Germany).

Immunohistochemistry

Pieces of the mammary gland of lactating rats on the 21st day of lactation ($n = 5$ in each dietary group) were snap frozen in OTC compound (4583, Miles Inc., Elkhart, USA) with liquid nitrogen prechilled isopentane and kept at -70°C until sectioned. Sections were cut, fixed and incubated with mouse monoclonal antibodies to detect T-lymphocytes (CD3+)(MCA 772), macrophages and dendritic cell expressing MHC class II (OX6+)(MCA46), dendritic cells (OX62+)(MCA1029), and activated dendritic cells expressing costimulatory molecules (CD86+)(MCA 1962) (Sero-tec, Oxford, UK) as previously described [23]. Cells were counted using a computer-supported image analysis system Leica Q500MC.

Statistical analysis

As the data were not normally distributed and standard deviations of the values of the groups were not equal nonparametric tests were used. Comparison between the animals that received OA orally (OA+) and those that did not receive (OA-) at each time point were analysed using the Mann-Whitney *U*-test. Comparisons between the groups that were given different diets were

Table 2. The fatty acid composition of serum phospholipids of the rat offspring at 3 weeks of age from dams fed diets with different ratios of n-6 and n-3 fatty acids

Fatty acids (mol%)	Dietary groups					
	n-3 (<i>n</i> = 8)		n-6/n-3 (<i>n</i> = 10)		n-6 (<i>n</i> = 10)	
12:0	1.6;	0.8–3.0*	1.3;	0.8–1.9*	1.0;	0.7–2.7*
14:0	2.7;	2.3–3.7*	2.1;	1.7–2.6†	2.0;	1.4–3.2†
16:0	26.4;	25.0–35.0*	24.1;	23.0–26.4†	24.7;	23.0–27.2†
16:1(n-7)	0.22;	0.18–0.42*	0.15;	0.12–0.24†	0.13;	0.1–0.21†
18:0	19.6;	16.7–20.1*	19.4;	18.0–20.9*	20.1;	18.1–21.1*
18:1(n-9)	4.9;	4.0–5.9*	3.7;	2.9–4.4†	3.7;	3.0–4.7†
18:2(n-6)	23.3;	8.5–24.9*	28.1;	25.2–30.0†	28.6;	21.2–31.5†
18:3(n-6)	0.06;	0.05–0.2*	0.07;	0.05–0.16*	0.12;	0.07–0.2†
18:3(n-3)	3.0;	1.4–5.1*	0.42;	0.2–0.6†	0.04;	0.02–0.05 ‡
20:2(n-6)	0.20;	0.16–0.22*	0.47;	0.4–0.52†	0.38;	0.32–0.46†
20:3(n-6)	0.53;	0.48–0.58*	0.4;	0.3–0.5†	0.42;	0.31–0.6†
20:4(n-6)	6.8;	5.8–11.9*	13.4;	11.9–14.5†	13.9;	12.7–17.8†
24:1(n-9)	0.6;	0.4–2.1*	0.4;	0.4–0.51†	0.4;	0.35–0.51†
20:5(n-3)	3.5;	0.8–4.5*	0.09;	0.06–0.1†	0.02;	0.01–0.04 ‡
22:6(n-3)	5.2;	4.1–10.8*	4.6;	3.9–5.1*	2.5;	1.9–3.8†
20:3(n-6)/20:4(n-6)	0.08;	0.04–0.09*	0.03;	0.02–0.04†	0.03;	0.02–0.05†
n-6	31.0;	21.4–33.4*	42.4;	40.6–42.9†	44.0;	39.8–45.2†
n-3	12.2;	10.4–13.2*	5.1;	4.4–5.6†	2.6;	1.9–3.7 ‡
n-6/n-3	2.5;	1.7–3.2*	8.2	7.3–9.6†;	16.9	10.6–23.5 ‡
Saturated FA	51.3;	50.7–57.2*	48.3;	47.1–49.7†	49.3;	47.6–52.2†
Monounsaturated FA	10.4;	8.7–14.0*	7.9;	6.2–9.1†	7.9;	6.3–9.9†
Polyunsaturated FA	43.1;	34.3–43.8*	47.5;	46.4–48.1†	46.7;	43.6–47.3†

Values are median; range. Different symbols (*†‡) indicate significant differences between dietary groups ($P < 0.05$) (multiple comparison with Kruskal–Wallis and Dunn's tests).

analysed using Kruskal–Wallis test followed by Dunn's test for nonparametric multiple comparisons. Values are given as median (range). A probability level of $<5\%$ was considered to be statistically significant. Different letters indicate significant differences between groups.

RESULTS

FA composition of serum PL

At three weeks of age there were significant changes in the FA composition of the serum PL in the offspring of the dams receiving different diets (Table 2). In the offspring of the dams on the n-3 diet the total levels of the n-6 FA in serum PL were significantly decreased, with an increase in the levels of myristic 14:0, palmitic 16:0, oleic 18:1(n-9), nervonic 24:1(n-9) acids and the total levels of the n-3 FA, compared to those of dams on the n-6/n-3 diet. In the offspring of the dams on the n-6 diet the levels of the n-3 FA in serum PL were significantly lower compared to the n-6/n-3 group. As a result the ratio of n-6/n-3 FA in serum PL were significant different in the n-3, the n-6/n-3 and the n-6 dietary group, being 2.5 (1.7–3.2), 8.2 (7.3–9.6) and 16.9 (10.6–23.5), respectively. In the n-3 dietary group the ratio of 20:3(n-6)/20:4(n-6) was significantly higher compared to the other dietary groups. In addition, the total levels of saturated and monounsaturated FA in serum PL were higher, while the levels of polyunsaturated FA were lower in the n-3 dietary group compared to other dietary groups.

Suppression of immune responses by oral administration of OA
DTH responses against OA, as well as against HSA were not significantly different between the dietary groups of the pups not exposed to OA (Fig. 2). In the pups exposed to OA DTH responses against OA were significantly lower in the n-3 and n-6 groups than in the n-6/n-3 dietary group, while DTH responses against HSA were significantly lower in the n-3 group only compared to that in the n-6/n-3 group. Oral administration of OA to lactating rats within the n-3 group led to a significant suppression of DTH responses against OA in the offspring, as well as against the bystander antigen HSA. Oral exposure to OA in the n-6 group resulted in reduced DTH responses to OA, but did not affect DTH responses against the bystander antigen HSA. In contrast, oral OA exposure of the dams fed the n-6/n-3 diet had no effect on the DTH responses against either OA or HSA in their offspring.

There were no significant differences in IgG anti-OA antibody levels, between the dietary groups (Fig. 3). In the offspring exposed to OA IgG and IgM anti-HSA antibody levels were significantly decreased in the n-3 group compared to the other dietary groups. IgM anti-OA and anti-HSA antibody levels in the offspring on the n-3 diet were significantly higher compared to that in the n-6/n-3 group, but were similar to that in the n-6 group. The offspring of the dams fed the n-3 diet, and exposed to OA orally via milk before immunization, had lower IgM and IgG anti-OA, as well as anti-HSA antibody levels than the offspring not exposed to OA orally. Oral administration of OA to the dams fed

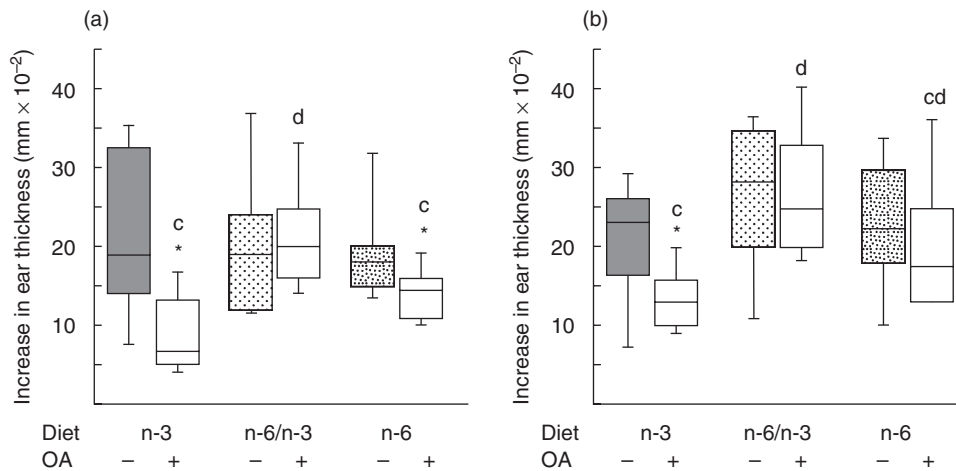


Fig. 2. DTH responses against (a) OA and (b) HSA in the offspring of the dams fed the diet with different ratios of n-6 to n-3 fatty acids and either exposed to OA orally (+) before immunization or not (-). The results are expressed as box plots that indicate median value of 9–10 animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Results with * are significantly different in the group exposed to OA orally, $P < 0.05$, from the group not exposed to OA within the dietary group. Different letters indicate significant differences between the dietary groups ($P < 0.05$) (multiple comparison with Kruskal–Wallis and Dunn's tests).

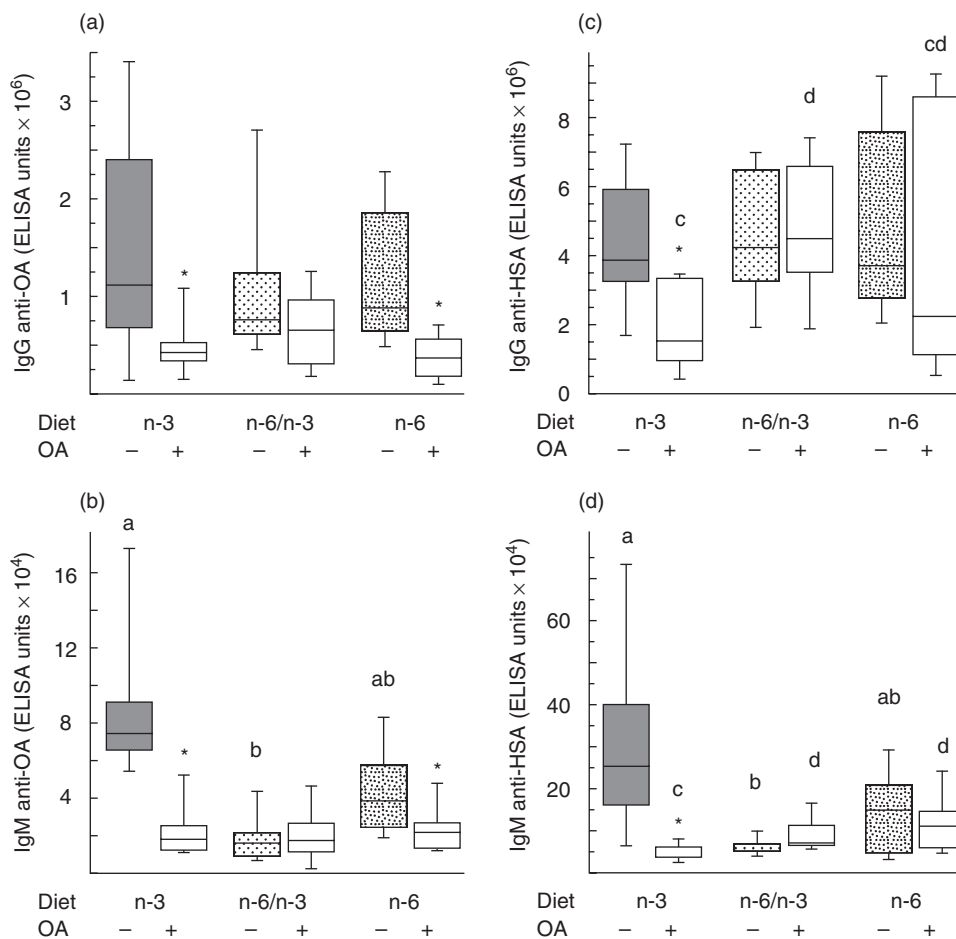


Fig. 3. Serum levels of IgG (a,c) and IgM (b,d) anti-OA (a,b) and anti-HSA (c,d) antibodies in the offspring of the dams fed the diet with different ratios of n-6 to n-3 fatty acids and either exposed to OA orally (+) before immunization or not (-). The results are expressed as box plots that indicate median value of 9–10 animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Results with * are significantly different in the animals exposed to OA orally, $P < 0.05$, from the animals not exposed to OA within the dietary group. Different letters (a and b in OA not exposed animals, c and d in OA exposed animals) indicate significant differences between the dietary groups ($P < 0.05$) (multiple comparison with Kruskal–Wallis and Dunn's tests).

the n-6 diet resulted in decreased IgG and IgM anti-OA antibody levels in their offspring, but did not change the antibody responses to HSA. The IgG and IgM anti-OA antibody levels, as well as anti-HSA antibody levels, were similar in the offspring of the dams on the n-6/n-3 diet whether treated orally with OA, or not (Fig. 3).

The serum IgE anti-OA antibody levels were very low and similar in the offspring of the dams fed the n-6/n-3 or n-6 diet whether given OA orally, or not (Fig. 4). The serum IgE anti-OA antibody levels tended to be higher in the offspring of the dams fed the n-3 diet that did not receive OA orally compare to that in the n-6 group. Oral administration of OA to the dams fed the n-

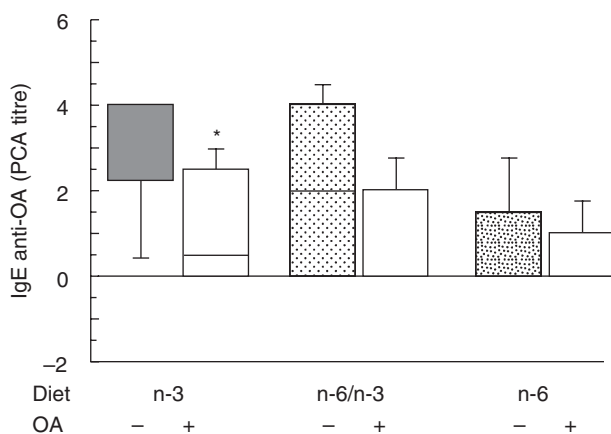


Fig. 4. Serum levels of IgE anti-OA antibodies in the offspring of the dams fed the diets with different ratios of n-6 to n-3 fatty acids and either exposed to OA orally before immunization or not. The results are expressed as box plots that indicate median value of 7–8 animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Results with * are significantly different in the group exposed to OA orally, $P < 0.05$, from the group not exposed to OA.

3 diet significantly decreased ($P < 0.05$) the serum IgE anti-OA antibody levels in their offspring.

Weights of draining lymph nodes

The weights of the draining PLN of the offspring of the dams fed the n-3 diet and exposed to OA orally were significantly lower ($P = 0.014$) than those of the immunized pups from the dams not given OA, being 6.0 (3.1–24.0) mg and 19.2 (7.0–43.6) mg, respectively. In the offspring of the dams on the n-6 diet the weight of the PLN were unrelated to whether or not OA was administered orally, being 18.6 (3.2–30.5) mg and 14.7 (6.3–28.0) mg, respectively. There was no significant difference ($P = 0.17$) in the weights of the PNL among the offspring of the dams fed the n-6/n-3 diet whether exposed to OA before immunization or not, 20.2 (6.4–31.8) mg and 12.4 (6.5–40.7) mg, respectively.

Immunohistochemical examination of the mammary gland tissue

The lactating rats fed the n-3 diet showed reduced numbers of macrophages and dendritic cells expressing MHC class II molecules in the mammary gland tissues compared to those in the two other dietary groups (Fig. 5). There were no differences in the number of T lymphocytes and dendritic cells in the mammary glands comparing the dietary groups (data not shown). The differences in the number of CD86+ cells in mammary gland tissue of the lactating rats fed the n-3 diet (3.4 cells/mm²; range 0.4–7.8), the n-6/n-3 diet (4.9 cells/mm²; range 1.6–21.9) and the n-6 diet (11.4 cells/mm²; range 1.2–33.0) were not significant (Kruskal–Wallis Test, $P = 0.59$).

DISCUSSION

In the present study the induction of immunological tolerance in the neonatal rats was affected by the ratio of n-6 to n-3 FA in the maternal diet. Failure to induce neonatal oral tolerance in the offspring was associated with the ratio of n-6/n-3 FA of 9 in the

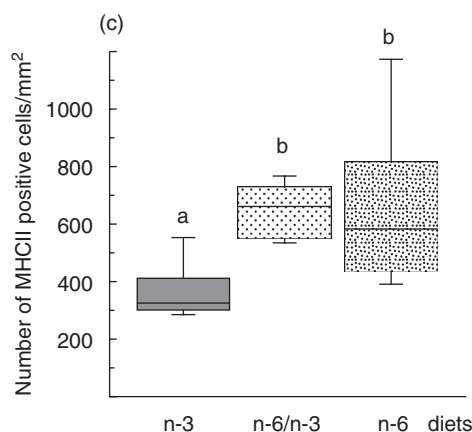
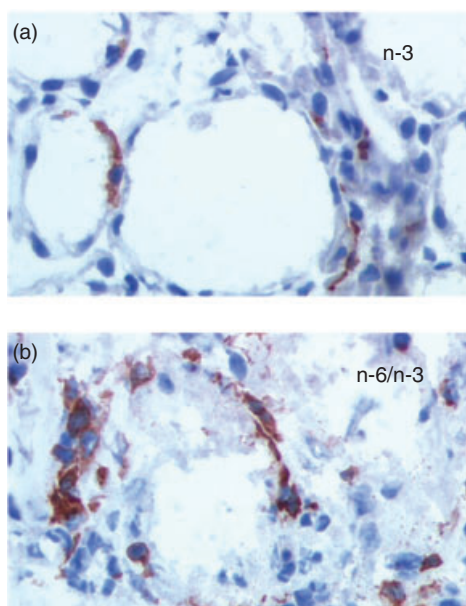


Fig. 5. MHC class II molecule positive cells (stained red) in mammary gland tissue of the lactating rats fed the diets with different ratios of n-6 to n-3 fatty acids. The results are expressed as box plots that indicate median value of 5 animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Different letters indicate significant differences between the dietary groups ($P < 0.05$) (multiple comparison with Kruskal–Wallis and Dunn's tests).

maternal diet. In addition, the present study suggests that the balance of the n-6/n-3 EFA in the diet might affect the mechanisms of neonatal oral tolerance.

It is well established that the FA composition of the maternal diet defines the FA levels in the maternal milk [6,24]. The FA composition of tissue and serum PL quickly adapt to that of the maternal milk in the suckling offspring [22,25], as also observed in the present study. The involvement of EFA and their derivatives in regulation of the immune cell functions [14,15] suggest that variation of the n-6/n-3 FA ratio in the milk might significantly affect the immune responsiveness of the offspring. In the offspring of the n-3 group the exposure to OA via the milk resulted in antigen-specific suppression of the DTH reaction to OA and suppressed levels of IgG and IgM anti-OA antibodies, confirming the induction of oral tolerance [10–12]. In addition, within the n-3 group the oral exposure to OA accompanied by reduction of DTH and IgG and IgM antibody responses to an unrelated antigen HSA, an effect known as bystander suppression. Several immunological mechanisms are known to contribute to induction and maintenance of oral tolerance like anergy, clonal selection and active suppression [26]. Occurrence of bystander suppression is associated with the existence of regulatory suppressor cells that are triggered by a specific antigen and responsible for the release of the antigen nonspecific suppressive cytokine TGF- β [27,28]. Consequently immune responses to other antigens in the close vicinity are diminished [29]. In rats made orally tolerant at adult age TGF- β producing cells appeared in the draining PLN after immunization and presumably suppressed the PLN swelling seen during the immune response [28]. We recorded that after the booster immunization with OA the draining PLN were less enlarged in the offspring of the dams fed the n-3 diet and orally treated with OA. We assume that in the offspring consuming the n-3 diet the oral tolerance was maintained and mediated at least partly by such an active suppression mechanism. In contrast, in the offspring of the dams fed the n-6/n-3 diet the induction of oral tolerance was not observed. Our results are consistent with the findings of Cinader *et al.* [16] which showed that the adult mice fed soybean oil failed to become tolerant to the heterologous gamma-globulin compared to the mice fed the diet with low content of polyunsaturated FA. The present study and that of Cinader *et al.* [16] differed in the route of tolerance induction, in the nature of tolerogen and in the age of animals. However, the loss of suppressor capacity might be due to similar changes in cell membrane composition or prostaglandin synthesis.

Further increase of the n-6 EFA in the maternal diet was again associated with the induction of oral tolerance after antigen exposure via the milk in the n-6 group of offspring. In contrast to the n-3 dietary group, the bystander suppression was not observed in the offspring receiving the n-6 diet, suggesting that the oral tolerance was probably mediated by an anergy mechanism, defined as a state of T lymphocyte unresponsiveness [30]. The predominant mechanism of tolerance is known to depend on the dose and nature of antigen, the frequency of antigen administration, the immunization route and the age of the host [19]. Recently, dietary levels of the n-6/n-3 FA has been shown to influence the mechanism of oral tolerance [18]. In mice fed n-3-rich fish oil the oral exposure to both low- and high-doses of OA suppresses anti-OA IgG2a antibody responses, while in mice fed n-6-rich borage oil only high-dose tolerance was induced [18]. In line, the present study suggests that the ratio of the n-6/n-3 EFA in the maternal diet might affect the mechanisms of neonatal oral tolerance.

The results suggest that the balance between the n-6 and n-3 EFA rather than an increase in the n-6 EFA levels might define the induction or failure of oral tolerance to OA in the offspring.

In the neonatal period the tolerance induction may be regulated by transmission of dietary antigen via breast milk. The extent and/or mode of antigen exposure and processing by both the mother and their offspring can be influenced by the protein delivery into the milk, the functionality of the intestinal barrier, and the maturation stage of the immune system. The FA composition of the mammary epithelial cell membranes has been shown to affect synthesis, secretion and intracellular transport of proteins in lactating mammary epithelial cells [31], which might influence both the quantity and quality of food antigens in the milk. The functionality of the intestinal barrier is also an important factor required for appropriate antigen exposure of neonatal rats. The balance of n-6/n-3 FA in maternal diet influences the ontogeny of the intestine and modifies the intestinal transport of nutrients in the suckling offspring [32]. Thus failure to induce oral tolerance in the offspring of the mothers receiving the n-6/n-3 diet might be due to inappropriate exposure to OA resulting from the altered antigen transfer to the milk and/or into the intestine.

The FA levels in the maternal diet might also alter immune responsiveness of neonates via effects on functions of the mammary glands that secrete multiple soluble immune factors and numerous immune cells [33]. Breast milk contains live neutrophils, macrophages, T and B lymphocytes [34,35]. Several studies have suggested that milk cells are taken up by the offspring and presumably capable of transferring immunological information [36]. We observed that the variations in the dietary EFA had no effects on the number of T lymphocytes and dendritic cells in the lactating mammary gland. In contrast, the number of macrophages and dendritic cell expressing MHC class II molecules were reduced in mammary gland of dams fed the n-3 diet, compared to that in the other two dietary groups. Long-chain n-3 FA have been shown to reduce MHC class II expression on dendritic cells and other antigen-presenting cells and decrease antigen presentation [37,38]. The n-3 FA also suppress the expression of adhesion molecules, costimulatory molecules and production of cytokines in immune cells [14,15]. That might result in changes of milk immune cell profile and functional activity or milk cytokine content and consequently be a mechanism in the modified neonatal immunological responsiveness to food antigen.

In conclusion, we found that the ratio of n-6/n-3 EFA in the maternal diet affected the induction of immunological tolerance to food antigen in rat offspring. The balance between n-6 and n-3 EFA in the milk, rather than increased n-6 EFA consumption might be one of the factors important for the induction, or failure of induction of oral tolerance in the neonatal period and contribute to the allergic sensitization to a food antigen.

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REFERENCES

- 1 Dunder T, Kuikka L, Turtinen J, Rasanen L, Uhari M. Diet, serum fatty acids, and atopic diseases in childhood. *Allergy* 2001; **56**:425–8.

- 2 Bolte G, Frye C, Hoelscher B, Meyer I, Wjst M, Heinrich J. Margarine consumption and allergy in children. *Am J Respir Crit Care Med* 2001; **163**:277–9.
- 3 Wakai K, Okamoto K, Tamakoshi A, Lin Y, Nakayama T, Ohno Y. Seasonal allergic rhinoconjunctivitis and fatty acid intake: a cross-sectional study in Japan. *Ann Epidemiol* 2001; **11**:59–64.
- 4 Sanders TA. Polyunsaturated fatty acids in the food chain in Europe. *Am J Clin Nutr* 2000; **71**:176S–8S.
- 5 Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* 2002; **56**:365–79.
- 6 Fidler N, Koletzko B. The fatty acid composition of human colostrum. *Eur J Nutr* 2000; **39**:31–7.
- 7 Oddy WH, Peat JK, de Klerk NH. Maternal asthma, infant feeding, and the risk of asthma in childhood. *J Allergy Clin Immunol* 2002; **110**:65–7.
- 8 Bergmann RL, Diepgen TL, Kuss O, Bergmann KE, Kujat J, Dudenhausen JW, Wahn U. Breastfeeding duration is a risk factor for atopic eczema. *Clin Exp Allergy* 2002; **32**:205–9.
- 9 Duchon K, Yu G, Bjorksten B. Atopic sensitization during the first year of life in relation to long chain polyunsaturated fatty acid levels in human milk. *Pediatr Res* 1998; **44**:478–84.
- 10 Kagnoff MF. Effects of antigen-feeding on intestinal and systemic immune responses. II. Suppression of delayed-type hypersensitivity reactions. *J Immunol* 1978; **120**:1509–13.
- 11 Hanson DG, Vaz NM, Maia LC, Hornbrook MM, Lynch JM, Roy CA. Inhibition of specific immune responses by feeding protein antigens. *Int Arch Allergy Appl Immunol* 1977; **55**:526–32.
- 12 Thomas HC, Parrott MV. The induction of tolerance to a soluble protein antigen by oral administration. *Immunology* 1974; **27**:631–9.
- 13 Mowat AM, Ferguson A. Hypersensitivity in the small intestinal mucosa. V. Induction of cell-mediated immunity to a dietary antigen. *Clin Exp Immunol* 1981; **43**:574–82.
- 14 Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. *Eur J Clin Nutr* 2002; **56** (Suppl. 3):S14–9.
- 15 Harbige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids* 2003; **38**:323–41.
- 16 Cinader B, Clandinin MT, Hosokawa T, Robblee NM. Dietary fat alters the fatty acid composition of lymphocyte membranes and the rate at which suppressor capacity is lost. *Immunol Lett* 1983; **6**:331–7.
- 17 Korotkova M, Telemo E, Hanson LA, Strandvik B. Modulation of neonatal immunological tolerance to ovalbumin by maternal essential fatty acid intake. *Pediatr Allergy Immunol* 2004; **15**:112–22.
- 18 Harbige LS, Fisher BA. Dietary fatty acid modulation of mucosally-induced tolerogenic immune responses. *Proc Nutr Soc* 2001; **60**:449–56.
- 19 Strobel S. Immunity induced after a feed of antigen during early life: oral tolerance v. Sensitisation *Proc Nutr Soc* 2001; **60**:437–42.
- 20 Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; **226**:497–509.
- 21 Pietsch A, Lorenz RL. Rapid separation of the major phospholipid classes on a single aminopropyl cartridge. *Lipids* 1993; **28**:945–7.
- 22 Korotkova M, Gabriellson B, Hanson L, Strandvik B. Maternal essential fatty acid deficiency depresses serum leptin levels in suckling rat pups. *J Lipid Res* 2001; **42**:359–65.
- 23 Karlsson MR, Kahu H, Hanson LA, Telemo E, Dahlgren UI. Neonatal colonization of rats induces immunological tolerance to bacterial antigens. *Eur J Immunol* 1999; **29**:109–18.
- 24 Saste MD, Carver JD, Stockard JE, Benford VJ, Chen LT, Phelps CP. Maternal diet fatty acid composition affects neurodevelopment in rat pups. *J Nutr* 1998; **128**:740–3.
- 25 Korotkova M, Gabriellson B, Lönn M, Hanson L, Strandvik B. Leptin levels in rat offspring are modified by the ratio of linoleic to alpha-linolenic acid in the maternal diet. *J Lipid Res* 2002; **43**:1743–9.
- 26 Weiner HL. Oral tolerance. immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infect* 2001; **3**:947–54.
- 27 Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci USA* 1992; **89**:421–5.
- 28 Lundin BS, Karlsson MR, Svensson LA, Hanson LA, Dahlgren UI, Telemo E. Active suppression in orally tolerized rats coincides with in situ transforming growth factor-beta (TGF-beta) expression in the draining lymph nodes. *Clin Exp Immunol* 1999; **116**:181–7.
- 29 Dahlman-Hoglund A, Dahlgren U, Ahlstedt S, Hanson LA, Telemo E. Bystander suppression of the immune response to human serum albumin in rats fed ovalbumin. *Immunology* 1995; **86**:128–33.
- 30 Komagata Y, Weiner HL. Oral tolerance. *Rev Immunogenet* 2000; **2**:61–73.
- 31 Ollivier-Bousquet M, Lavialle F, Guesnet P, Rainteau D, Durand G. Lipid-depleted diet perturbs membrane composition and intracellular transport in lactating mammary cells. *J Lipid Res* 1997; **38**:913–25.
- 32 Jarocka-Cyrta E, Perin N, Keelan M, Wierzbicki E, Wierzbicki T, Clandinin MT, Thomson AB. Early dietary experience influences ontogeny of intestine in response to dietary lipid changes in later life. *Am J Physiol* 1998; **275**:G250–8.
- 33 Hanson LA, Korotkova M, Telemo E. Breast-feeding, infant formulas, and the immune system. *Ann Allergy Asthma Immunol* 2003; **90**:59–63.
- 34 Keeney SE, Schmalstieg FC, Palkowetz KH, Rudloff HE, Le BM, Goldman AS. Activated neutrophils and neutrophil activators in human milk. increased expression of CD11b and decreased expression of 1-selectin. *J Leukoc Biol* 1993; **54**:97–104.
- 35 Bertotto A, Gerli R, Fabietti G, Crupi S, Arcangeli C, Scalise F, Vaccaro R. Human breast milk T lymphocytes display the phenotype and functional characteristics of memory T cells. *Eur J Immunol* 1990; **20**:1877–80.
- 36 Zhou L, Yoshimura Y, Huang Y, Suzuki R, Yokoyama M, Okabe M, Shimamura M. Two independent pathways of maternal cell transmission to offspring: through placenta during pregnancy and by breast-feeding after birth. *Immunology* 2000; **101**:570–80.
- 37 Sanderson P, MacPherson GG, Jenkins CH, Calder PC. Dietary fish oil diminishes the antigen presentation activity of rat dendritic cells. *J Leukoc Biol* 1997; **62**:771–7.
- 38 Hughes DA, Pinder AC, Piper Z, Johnson IT, Lund EK. Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes. *Am J Clin Nutr* 1996; **63**:267–72.