

# Modulation of neonatal immunological tolerance to ovalbumin by maternal essential fatty acid intake

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The present study examines whether dietary essential fatty acid (EFA) intake influences the induction of oral tolerance to ovalbumin (OA) in neonatal and adult rats. During late gestation and throughout lactation Sprague–Dawley rats were fed a diet supplemented (S) with EFA (7% soybean oil), or a diet deficient (D) in EFA (7% hydrogenated lard). The rat offspring were subsequently exposed to OA either via the milk at 10–16 days (neonatal rats), or as adults via the drinking water at 7–9 wk of age. Oral administration of OA to the adult rats lead to suppression of the delayed-type hypersensitivity (DTH) reactivity and IgG antibody response against OA, which was not influenced by their diets. In the offspring of the dams fed the D diet antigen exposure via the milk resulted in suppression of the serum antibody levels and DTH reaction against OA indicating induction of oral tolerance. Higher transforming growth factor beta (TGF- $\beta$ ) mRNA levels in the draining lymph nodes suggested this to be mediated by regulatory T cells. In contrast, OA exposure of the dams fed the S diet did not result in a suppressed OA response of their offspring. Thus, the quality of FA ingested by the mother may have effects on the development of immunological tolerance to dietary antigens in the offspring. Our results might have importance for the understanding of the increase in allergy related to the Western type of diet.

**Marina Korotkova<sup>1</sup>, Esbjörn Telemo<sup>2</sup>,  
Lars Å. Hanson<sup>2</sup> and Birgitta  
Strandvik<sup>1</sup>**

Departments of <sup>1</sup>Pediatrics and <sup>2</sup>Clinical Immunology,  
Göteborg University, Göteborg, Sweden

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Marina Korotkova, Department of Pediatrics,  
Göteborg University, SE 41685 Göteborg, Sweden  
Tel.: +46 31 343 52 30  
Fax: +46 31 21 70 23  
E-mail: marina.korotkova@cmm.ki.se

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During the neonatal period the gastrointestinal tract is exposed to a wide variety of microbial and food-related antigens. Oral exposure to foods or other foreign protein antigens usually results in a state of specific immunological hyporesponsiveness, known as oral tolerance (1). A breakdown or failure of the development of oral tolerance to components of foods may lead to allergic diseases. Factors important for the induction or failure of oral tolerance in the neonatal period are poorly understood. In neonatal rodents oral exposure to antigen can induce tolerance or priming depending on the age at the first antigen administration (1, 2). Antigen feeding before 7 days of age induces priming of subsequent humoral and delayed-type hypersensitivity (DTH) responses (2), probably because of

immunological immaturity (3). Starting from 7 days of age, oral administration of antigen leads to induction of oral tolerance of antibody and cell-mediated immune responses (1, 2), illustrating the relative maturity of the immune system at this age.

The breast milk contains numerous factors, which may promote the development of the infant's immune system (4), including essential fatty acids (EFA). The EFA, linoleic 18:2(n-6) and  $\alpha$ -linolenic 18:3(n-3) acids and their metabolites as structural membrane elements and functional modulators, affect immune responsiveness through alteration of the functions of lymphocytes and macrophages (5). The maternal diet is the most important variable determining the FA composition of membrane phospholipids

(PL) of the offspring (6). Thus, variation in the EFA intake in the maternal diet might significantly modulate neonatal development of immunological tolerance and gastrointestinal sensitization to food antigens. Controversial results of recent studies on the protective role of breastfeeding against allergic diseases (7, 8) might be linked to variations in the FA composition of breast milk. Epidemiological studies suggest that the present increase in unsaturated fat consumption is associated with an increased prevalence of atopic diseases (9, 10). A restricted intake of dietary n-6 EFA has been shown to weaken ongoing inflammatory responses in a model of food-sensitive enteropathy in mice (11) and to decrease the severity of atopic diseases in humans (12). However, whether the intake of dietary EFA affects the induction of oral tolerance has not been investigated previously.

The present study examines whether the intake of dietary EFA influences the induction of oral tolerance to the food antigen ovalbumin (OA) in neonatal and adult rats. To induce oral tolerance in adult rats a continuous exposure of OA in drinking water was used. 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 (1). After oral exposure to OA and following immunization with this antigen an antigen-specific suppression of antibody responses and cell-mediated immune responses such as DTH was evaluated. In orally tolerized adult animals a suppressed inflammatory response of the draining lymph node has been observed presumably because of the presence of transforming growth factor beta (TGF- $\beta$ ) producing cells in the draining lymph node (13). Therefore, we have investigated an enlargement of and TGF- $\beta$  mRNA expression in the draining lymph nodes in orally tolerized neonatal rats.

## Material 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 females were assigned to one of two groups, receiving either an EFA-supplemented (S) or an EFA-deficient (D) diet, containing mainly saturated

FA (AnalyCen, Lidköping, Sweden). The diets differed only by their lipid composition: 7% soybean oil for the S diet and 7% hydrogenated lard for the D diet. The composition of the two diets is given in Table 1. The offspring were kept with their mothers until weaning at 21 days. The study were approved by the Animal Ethics Committee of Göteborg University.

### OA administration and immunization of adult rats

After weaning the offspring received the same diet as the dams. The 7-wk-old female rats of each dietary group were divided into two subgroups ( $n = 8$  in each) and caged in a group of four animals. In order to induce oral tolerance against OA, the rats were given OA (grade V, Sigma Chemical Co., St Louis, MO, USA) via the drinking water (corresponding to an approximate intake of 80 mg OA/day for each animal) for 2 wk, OA(+) or just water as control, OA(–) (Fig. 1). Two weeks after the removal of the OA in the water all rats were immunized subcutaneously (s.c.) in the legs with 100  $\mu$ g OA mixed with 100  $\mu$ g human serum albumin (HSA)

Table 1. Composition of essential fatty acid (EFA)-supplemented (S) and EFA-deficient (D) diets

| Diet (weight, %)     | S    | D    |
|----------------------|------|------|
| Casein               | 20.0 | 20.0 |
| Potato starch        | 54.0 | 54.0 |
| Glucose              | 10.0 | 10.0 |
| Cellulosal flour     | 4.0  | 4.0  |
| Mineral mix*         | 4.0  | 4.0  |
| Vitamin mix†         | 1.0  | 1.0  |
| Soybean oil          | 7.0  |      |
| Hydrogenated lard    |      | 7.0  |
| Fatty acids (mol, %) |      |      |
| 12:0                 | 0.1  | 0.2  |
| 14:0                 | 0.2  | 0.3  |
| 16:0                 | 10.8 | 5.4  |
| 16:1                 | 0.1  | 0    |
| 18:0                 | 9.0  | 41.0 |
| 18:1(n-9)            | 18.9 | 0.7  |
| 18:2(n-6)            | 45.7 | 0    |
| 20:0                 | 1.7  | 9.9  |
| 18:3(n-3)            | 8.1  | 0    |
| 20:2                 | 0.1  | 0    |
| 22:0                 | 5.0  | 41.4 |
| 24:0                 | 0.2  | 1.2  |

\*Mineral mixture containing (weight, %):  $\text{KH}_2\text{PO}_4$  (34.1);  $\text{CaCO}_3$  (35.9); KCl (2.5); NaCl (18);  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$  (5.1);  $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$  (3.3); MnO (0.27);  $\text{Cu}_2\text{C}_6\text{H}_4\text{O}_7 \cdot 2.5\text{H}_2\text{O}$  (0.06);  $\text{Zn}_3(\text{C}_6\text{H}_5\text{O}_7)_2 \cdot 2\text{H}_2\text{O}$  (0.04);  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.002);  $\text{KAl}(\text{SO}_4)_2 \cdot 2\text{H}_2\text{O}$  (0.008); NaF (0.025);  $\text{KIO}_3$  (0.009);  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  (0.002);  $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$  (0.001);  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.001).

†Vitamin mixture containing: A, 11.9 IE/g; D<sub>3</sub>, 1.5 IE/g; B<sub>1</sub>, 4 mcg/g; B<sub>2</sub>, 12 mcg/g; B<sub>6</sub>, 5 mcg/g; Ca-pantotenate 45%, 11 mcg/g; niacin, 40 mcg/g; B<sub>12</sub>, 0.02 mcg/g; K<sub>3</sub>, 7.75 mcg/g; biotin 2%, 3 mcg/g; C, 500 mcg/g; inositol, 30 mcg/g; E, 42 mcg/g; choline chloride 50%, 1 mg/g; folic acid, 0.5 mcg/g.

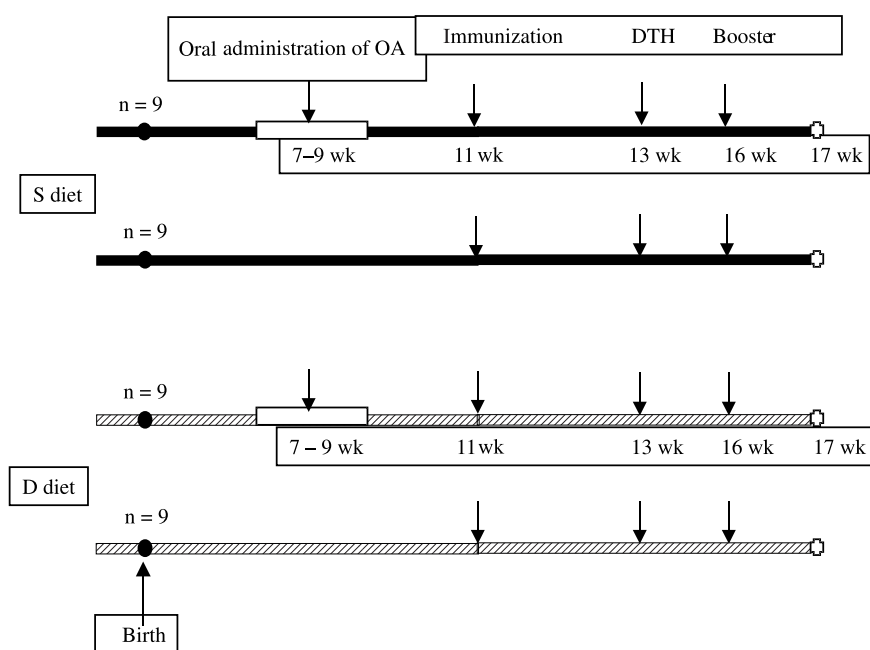


*Fig. 1.* The protocol for the immunization of adult rats fed a diet supplemented (S) with or deficient (D) in essential fatty acids. During late gestation and throughout lactation rat dams were fed the S diet or the D diet and after weaning the offspring received the same diet as the dams. The rat offspring were exposed to ovalbumin (OA+) via the drinking water at 7–9 wk of age or just water as control (OA–). Two weeks older rats were immunized with OA mixed with human serum albumin (HSA). Delayed-type hypersensitivity (DTH) reactions were tested 2 wk after the immunization. Five weeks after the primary immunization rats were boosted with OA mixed with HSA. One week after the booster dose the animals were killed.

(Fraction V, Sigma) in complete Freund's adjuvant. DTH reactions were tested 2 wk after the immunization. At week 5 after the primary immunization rats were boosted intraperitoneally with 100  $\mu$ g OA mixed with 100  $\mu$ g HSA in phosphate-buffered saline (PBS). One week after the booster dose the animals were killed using CO<sub>2</sub>. Blood samples were obtained from the tip of the tail before immunization and weekly thereafter and sera were kept at  $-20^{\circ}\text{C}$ .

OA administration to the dams and immunization of the neonatal rats

Pups randomized from each litter were used for each dietary group ( $n = 9$  per group). In order to induce oral tolerance against OA in the neonatal rats, the dams of each dietary group were given either drinking water with OA (200 mg/day for dam) or just water as control on days 10–16 of lactation (Fig. 2). At 3 wk of age all offspring were weaned onto the S diet and immunized s.c. in the legs with 100  $\mu$ g of OA in complete Freund's adjuvant. DTH reactions were tested 2 wk after the immunization. Three weeks after the primary immunization the rats were boosted s.c. with 100  $\mu$ g OA in incomplete Freund's adjuvant. One week after the booster dose the animals were killed using CO<sub>2</sub>. Blood samples were obtained from the tip of the tail before immunization and weekly thereafter and sera were kept at  $-20^{\circ}\text{C}$ . The draining popliteal lymph nodes (PLN) were removed, weighed with an analytical top loader balance, placed into the RNeasy lysis buffer (Qiagen, Crawley, UK) and

stored at  $-20^{\circ}\text{C}$ . Small intestine from non-immunized pups at 3 wk of age was removed. Pieces of the jejunum were rinsed with cold saline and the mucosa was scraped with a plastic loop and stored at  $-20^{\circ}\text{C}$ .

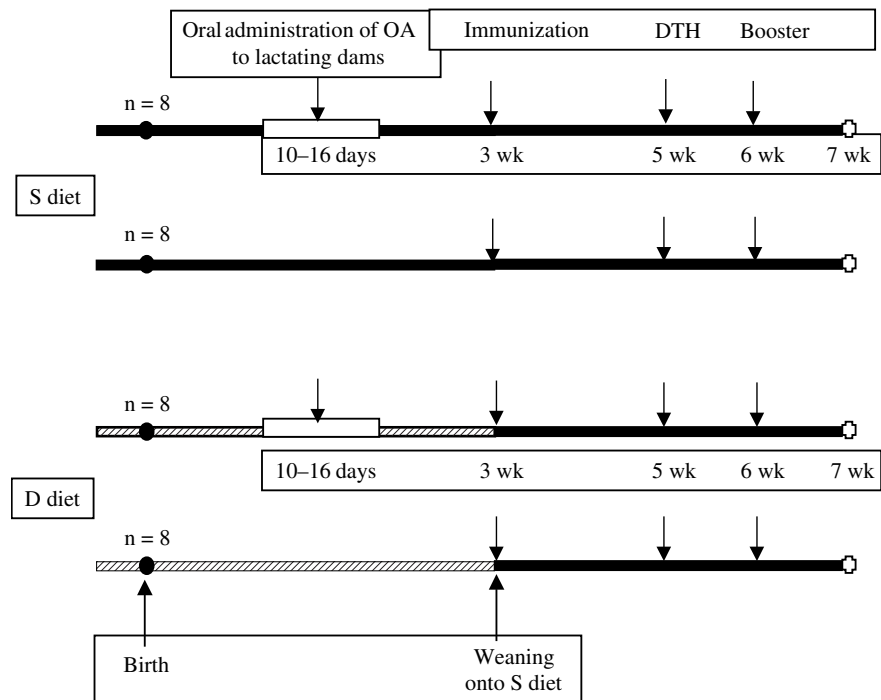
#### Fatty acid analysis

Total lipids of serum or jejunal mucosa were extracted according to Folch et al. (14), fractionated on a single SEP-PAK aminopropyl cartridge (Waters Corp., Milford, MA, USA) (15) 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 (Wilmington, DE, USA) according to the method described previously (16). 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). The ratio of 20:3( $n-9$ ) to 20:4( $n-6$ ) was used to define the deficiency state: a ratio of  $>0.4$  was considered as the biochemical criterion of EFA deficiency (17).

#### Antibody determination

Serum anti-OA antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as follows. Microtitre plates (MIC-2000, Dynatech, Alexandria, VA, USA) were coated overnight with 5  $\mu$ g/ml OA in PBS. The serum samples were optimally diluted in PBS-Tween (0.05%),

**Fig. 2.** The protocol for the immunization of neonatal offspring of dams fed the diet supplemented (S) with or deficient (D) in essential fatty acids. During late gestation and throughout lactation rat dams were fed the S diet or the D diet and all the offspring were weaned onto the S diet. To expose the rat offspring to ovalbumin (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 wk of age the offspring were immunized with OA. Delayed-type hypersensitivity (DTH) reactions were tested 2 wk after the immunization. Three weeks after the primary immunization the rats were boosted with OA. One week after the booster dose the animals were killed.



added to the plates and incubated for 3 h at room temperature. Rabbit anti-rat IgG antibodies, dilution: 1/10000 (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, PA, USA) 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 for 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 Labs, MacLean, VA, USA) after 60 min. The plates were washed with PBS-Tween between the incubations. The antibody levels were expressed in arbitrary ELISA units calculated from a standard curve obtained with a pool of hyperimmune serum.

#### Delayed-type hypersensitivity

The DTH responses to OA and HSA were tested 2 wk after the primary immunization by injecting 50 µg of antigen in 20 µl 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).

#### RNA extraction and analysis by RT-PCR

Total RNA was isolated from the lymph nodes of each individual rat with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and used for analysis of cytokine messenger RNAs (mRNAs) by real-time polymerase chain reaction (RT-PCR). The RNA samples were treated with DNase (DNA-free™, Ambion) according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (OD<sub>260</sub>) and its integrity was verified by agarose gel electrophoresis, with visualization by ethidium bromide (EtdBr) staining.

Synthesis of cDNA was performed according to the method described previously (18). The cDNA was stored at -70°C. Multiplex relative RT-PCR was used for the analysis of differences in mRNA abundance. The cytokine gene was co-amplified with invariant endogenous control. The cDNA was amplified by PCR using specific primers for the rat IL-4 cDNA (19). The primer pairs: 5'-ACC TTG CTG TCA CCC TGT TCT GC-3' (nucleotides 872–894) and 5'-GTT GTG AGC GTG GAC TCA TTC ACG-3' (nucleotides 2361–2384) (Scandinavian Gene Synthesis AB, Köping, Sweden), generated a single 352 bp product. Rat interferon gamma (IFN-γ) Amplimer Set and rat TGF-β Amplimer Set (Clontech Laboratories, Inc., Palo Alto, CA, USA) were used for gene-specific amplification. The annealing temperature and the number of cycles were optimized for each primer pair. QuantumRNA

18S internal standard (Ambion) was used as internal control and generated a single 489 bp product. Linear range and optimal ratio of 18S primers: competitors were determined. The PCR was carried out according to the method described previously (18). In the negative control the reverse transcriptase was omitted for each sample resulting in no bands after RT-PCR.

The PCR products were separated on 2% EtdBr agarose gel and were subsequently visualized and quantified using IPLab Gel Scientific Image processing (Signal Analytics, Vienna, VA, USA). The intensity obtained for cytokine amplicon was related to that of 18S in each individual sample.

#### Immunohistochemistry

Pieces of the jejunum were snap frozen in Tissue-Tek OTC compound (4583, Miles Inc., Elkhart, IN, USA) with liquid nitrogen pre-chilled isopentane and kept at  $-70^{\circ}\text{C}$  until sectioned. Sections were cut, fixed and incubated with mouse monoclonal antibodies directed against major histocompatibility complex (MHC) class II (MCAP46),  $\text{TCR}\alpha\beta$  (MCA453), CD4 (MCA55) and CD8 (MCA48) (Serotec, Oxford, UK) as previously described (20). Cells were counted using a computer-supported image analysis system Leica Q500MC (Leica Cambridge Ltd., Cambridge, UK). The MHC class II and CD4 expression in the villi were recorded as the stained tissue area divided by the whole measured area  $\times 100\%$ .

#### Statistical analysis

As the data were not normally distributed and s.d. of the values of the groups were not equal a non-parametric test was used. Comparison between the animals that received OA orally (OA+) and those that did not received (OA-) at each time point were analysed using the Mann-Whitney *U*-test. Values are given as median (range). A probability level of  $<5\%$  was considered to be statistically significant.

## Results

#### Adult rats

**Fatty acid composition of the serum phospholipids.** The levels of linoleic 18:2(n-6),  $\alpha$ -linolenic 18:3(n-3), arachidonic 20:4(n-6) and docosahexaenoic 22:6(n-3) acids in serum PL in the adult rats receiving the D diet were significantly less than in the group, which received the S diet ( $p < 0.01$ ) (Table 2). The levels of oleic 18:1(n-9), eicosatri-

Table 2. Fatty acids composition of the serum phospholipids of the adult rats receiving the essential fatty acid (EFA)-supplemented (S) or EFA-deficient (D) diet

| Fatty acids (mol, %) | S diet (n = 8)   | D diet (n = 8)     |
|----------------------|------------------|--------------------|
| 12:0                 | 0.1 (0.1–0.2)    | 0.3 (0.2–0.9)**    |
| 14:0                 | 0.15 (0.1–0.2)   | 0.3 (0.3–0.4)**    |
| 16:0                 | 17.0 (15.6–20.1) | 18.5 (16.7–20.0)   |
| 16:1(n-7)            | 0.1 (0.1–0.1)    | 2.1 (1.5–2.3)**    |
| 18:0                 | 36.9 (33.8–39.6) | 33.0 (28.3–35.4)** |
| 18:1(n-9)            | 2.8 (2.5–3.5)    | 14.6 (12.1–20.7)** |
| 18:2(n-6)            | 15.0 (13.4–16.2) | 1.7 (1.3–2.8)**    |
| 20:0                 | 0.2 (0.2–0.3)    | 0.2 (0.2–0.2)      |
| 18:3(n-3)            | 0.1 (0.1–0.1)    | 0.1 (0.0–0.0)**    |
| 20:2(n-6)            | 0.3 (0.3–0.3)    | 0.2 (0.1–0.9)      |
| 20:3(n-9)            | 0.0 (0.0–0.0)    | 20.8 (17.9–23.7)** |
| 22:0                 | 1.7 (1.4–2.0)    | 1.2 (1.1–1.3)**    |
| 20:4(n-6)            | 17.4 (15.3–18.5) | 2.0 (1.1–2.8)**    |
| 24:0                 | 1.7 (1.4–1.9)    | 1.4 (1.3–1.6)*     |
| 24:1(n-9)            | 0.8 (0.7–3.0)    | 0.5 (0.4–0.7)**    |
| 22:6(n-3)            | 5.0 (4.5–5.8)    | 1.5 (0.8–2.1)**    |
| n-6/n-3              | 6.5 (5.4–7.0)    | 2.9 (2.1–3.9)**    |
| 18:1(n-9)/18:2(n-6)  | 0.2 (0.2–0.3)    | 9.3 (4.3–15.9)**   |
| 20:3(n-9)/20:4(n-6)  | 0.0 (0.0–0.0)    | 11.3 (7.3–16.3)**  |

Values are median (range).

Results with \* and \*\* are significantly different in the group receiving D diet,  $p < 0.05$  and  $<0.01$ , respectively from the group receiving S diet.

enoic 20:3(n-9) and palmitoleic 16:1(n-7) acids were as compensation higher in the serum PL in the adult rats receiving the D diet than in the S group ( $p < 0.01$ ). The biochemical index of the EFA nutritional deficit, the ratio of 20:3(n-9) to 20:4(n-6) in the D groups was above the empiric value. The ratio of n-6/n-3 FA in serum PL of the rats on the S diet was significantly higher compared with that of the rats on the D diet.

**Suppression of immune responses by oral administration of OA.** Oral administration of OA to adult rats resulted in a significant decrease in the DTH-reaction to OA ( $p < 0.01$ ) compared with the age-matched OA(–) group, irrespective of their diet (Fig. 3). The oral administration of OA to the animals, which received the S or the D diets, also leads to a suppressed DTH-reaction to the bystander antigen HSA ( $p < 0.01$ ).

Starting from the third week after immunization the rats exposed to OA orally before immunization had significantly lower anti-OA IgG antibody levels than rats non-exposed to OA unrelated to the diets ( $p < 0.05$ ) (Fig. 4). The anti-HSA IgG antibody levels of the OA(+) group fed the S diet were lower compared with the OA(–) group only at 6 wk after immunization ( $p < 0.05$ ). However, in the rats fed the D diet the anti-HSA IgG antibody levels were diminished ( $p < 0.05$ ) in the tolerant animals from 3 wk after immunization.

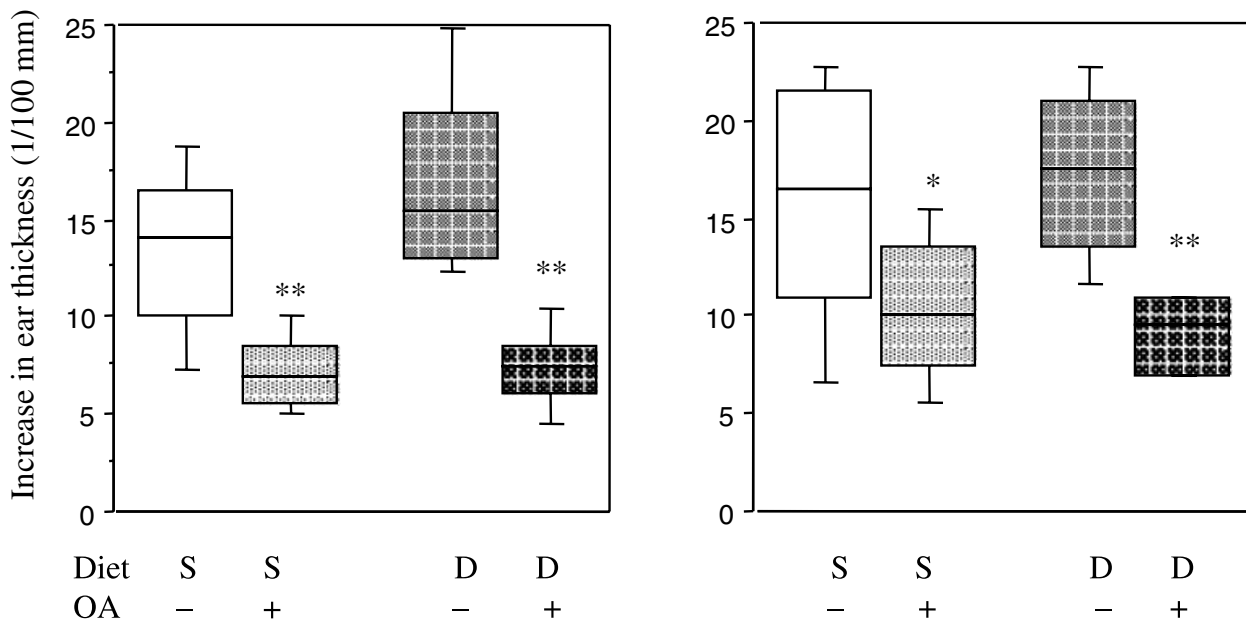


Fig. 3. Delayed-type hypersensitivity (DTH) reactions against ovalbumin (OA) (a) and human serum albumin (HSA) (b) in adult rats fed a diet supplemented with (S) or deficient (D) in essential fatty acids and exposed to OA orally (+) or not exposed (-) before immunization. The results are expressed as box plots that indicate median value of eight animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Results with \* and \*\* are significantly different in the group exposed to OA orally,  $p < 0.05$  and  $p < 0.01$ , respectively, from the group not exposed to OA.

#### Neonatal rats

**Fatty acid composition of the serum and jejunal mucosa phospholipids.** The FA composition of the serum PL, as well as the jejunal PL, was changed in the offspring of the D group at 3 wk of age compared with that in the S group of offspring (Table 3). The ratio of 20:3(n-9) to 20:4(n-6) in the serum PL and in the jejunal PL in the D group was above the empiric value, confirming the EFA nutritional deficit. The levels of 18:2(n-6) and 18:3(n-3) in the PL of both serum and jejunal mucosa in the neonatal rats receiving the D diet were significantly lower compared with the S group. In the group that received the D diet the levels of 20:4(n-6) in the serum PL were significantly lower than in the group that received the S diet ( $p < 0.05$ ). However, the levels of 20:4(n-6) in the jejunal mucosa PL of the offspring from the dams on the D diet did not differ from those in the S group of pups, while the proportion of 22:6(n-3) was significantly increased in the group fed the D diet compared with the group fed the S diet. The ratios of n-6/n-3 FA in serum as well as jejunal PL of the rats on the S diet were significantly higher compared with those of the rats on the D diet.

**Suppression of immune responses by oral administration of OA.** Oral administration of OA to lactating rats fed the D diet resulted in a

significant decrease in the DTH reaction to OA ( $p < 0.05$ ) in their offspring compared with the age-matched group not exposed to OA (Fig. 5). The specific DTH responses against OA were similar in the offspring of the dams fed the S diet whether exposed to OA during lactation or not.

The anti-OA IgG and IgM antibody levels were not significantly different in the offspring of the dams fed the S diet whether receiving OA via the milk or not (Fig. 6). In the offspring of the dams fed the D diet and orally provided with OA, the anti-OA IgG and IgM antibody levels were significantly decreased ( $p < 0.05$ ) compared with those of the offspring not exposed to OA.

**Immunohistochemical examination of the small intestine.** In the small intestinal villi of non-immunized adult rats, whether fed the S, or the D diet, the number of CD8-positive cells were similar [429 (235–630) and 375 (294–499) cells/mm<sup>2</sup>, respectively], as well as the percentage of cells which expressed MHC class II molecules [24.6 (17.4–37.7) and 26.5 (16.0–34.6)%, respectively]. There were no significant differences in the number of CD4-, and TCR $\alpha$ / $\beta$ -positive cells (data not shown). The expression of MHC class II molecules in the small intestinal villi of the non-immunized neonatal rats of the dams fed the

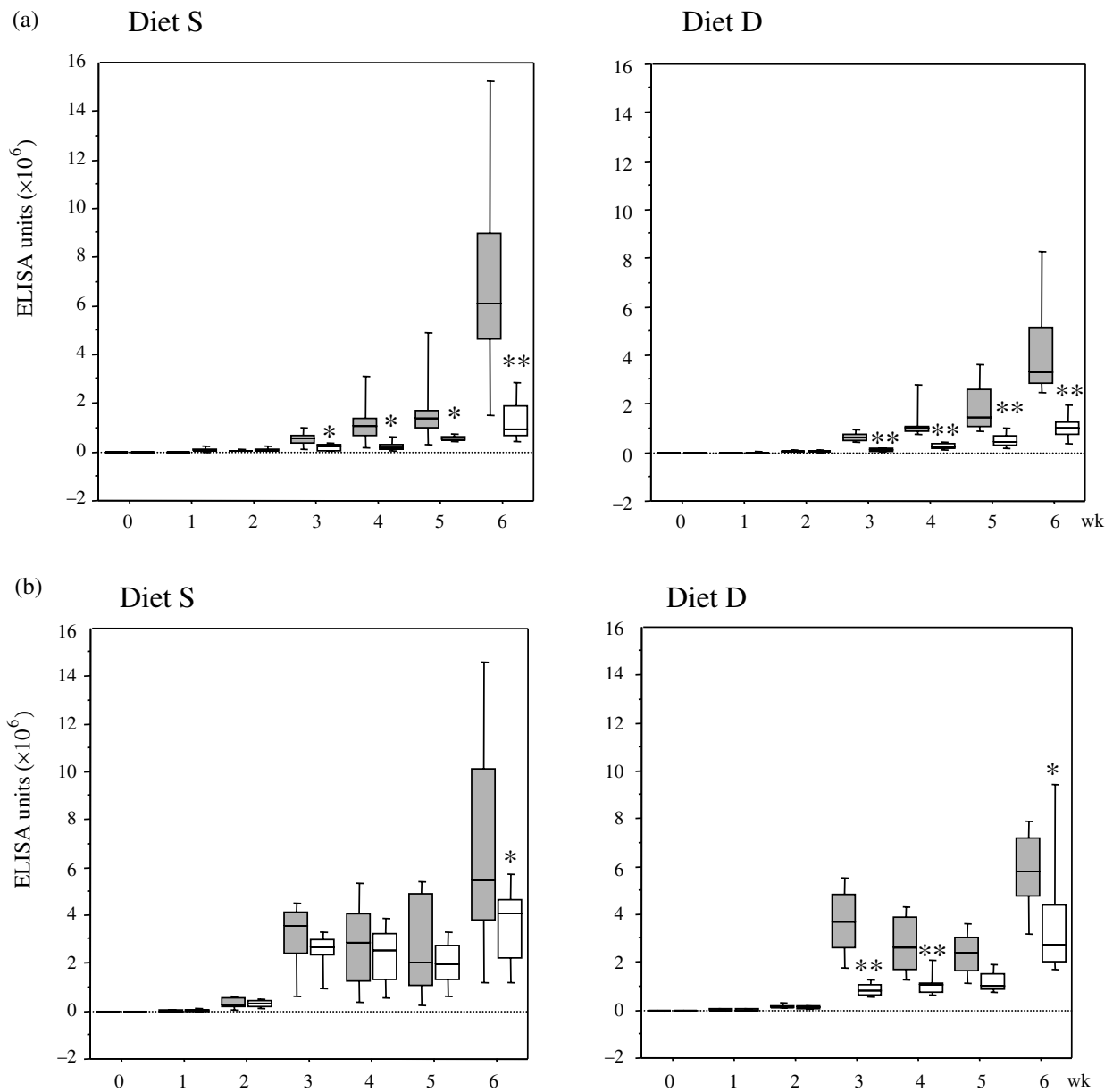


Fig. 4. Serum levels of IgG anti-ovalbumin (OA) (a) and anti-human serum albumin (HSA) (b) antibodies in adult rats fed the diet supplemented (S) with or deficient (D) in essential fatty acids and exposed to OA orally (unfilled boxes) or not exposed (filled boxes) before immunization. The results are expressed as box plots that indicate median value of nine animals, lower and upper quartiles. Whiskers show the 10th and 90th percentiles. Results with \* and \*\* are significantly different in the group exposed to OA orally,  $p < 0.05$  and  $p < 0.01$ , respectively, from the group not exposed to OA.

S or the D diet was similar [7.9 (6.1–15.1) and 10.1 (5.4–19.7)%, respectively]. However, the number of CD8-positive T-cells was higher in the neonatal rats from the dams on the D diet than in those from the dams on the S diet [328 (278–385) and 227 (195–299) cells/mm<sup>2</sup>, respectively;  $p < 0.05$ ].

*TGF- $\beta$  expression in draining popliteal lymph nodes.* The mean weights of the draining PLNs

of the offspring from the dams fed the D diet and exposed to OA orally were significantly lower ( $p < 0.05$ ) than those of the immunized pups from the dams not given OA [9.4 (7.2–22.0) and 15.0 (8.7–23.7) mg, respectively]. In the offspring of the dams fed the S diet the weight of the PLNs were similar in both groups, unrelated to whether or not OA was administered to the dams [12.1 (6.2–22.7) and 14.2 (6.2–24.7) mg, respectively].

Table 3. Fatty acid composition of the serum and jejunal mucosa phospholipids of the neonatal rats at 3 wk of age from dams receiving the essential fatty acid (EFA)-supplemented (S) or EFA-deficient (D) diet

| Fatty acids (mol, %) | Serum            |                    | Jejunal mucosa   |                    |
|----------------------|------------------|--------------------|------------------|--------------------|
|                      | S diet (n = 6)   | D diet (n = 6)     | S diet (n = 6)   | D diet (n = 6)     |
| 12:0                 | 0.1 (0.1–0.2)    | 0.3 (0.2–0.3)**    | 0.3 (0.2–0.4)    | 0.4 (0.2–0.5)      |
| 14:0                 | 1.0 (0.9–1.1)    | 1.6 (1.3–2.0)**    | 1.1 (0.9–1.3)    | 1.9 (1.5–2.1)**    |
| 16:0                 | 25.8 (23.7–29.6) | 29.6 (27.3–32.3)*  | 20.0 (18.7–20.4) | 26.2 (25.0–27.1)** |
| 16:1(n-7)            | 0.1 (0.1–0.1)    | 1.3 (1.0–1.9)**    | 0.1 (0.1–0.1)    | 1.3 (1.1–2.2)**    |
| 18:0                 | 22.5 (22.0–24.9) | 17.5 (16.5–18.6)** | 24.7 (24.5–25.4) | 21.0 (20.6–21.2)** |
| 18:1(n-9)            | 3.0 (2.8–3.3)    | 17.9 (14.4–21.0)** | 3.7 (3.4–3.8)    | 14.1 (11.5–18.3)** |
| 18:2(n-6)            | 28.5 (27.0–29.8) | 9.1 (7.1–11.2)*    | 32.1 (30.6–33.7) | 10.3 (9.1–13.4)**  |
| 20:0                 | 0.2 (0.2–0.2)    | 0.1 (0.1–0.1)**    | 0.5 (0.4–0.6)    | 0.4 (0.3–0.4)*     |
| 18:3(n-3)            | 0.2 (0.2–0.3)    | 0.1 (0.1–0.2)*     | 0.4 (0.4–0.4)    | 0.2 (0.1–0.3)**    |
| 20:2(n-6)            | 0.4 (0.4–0.5)    | 0.4 (0.3–0.5)      | 0.4 (0.4–0.5)    | 0.3 (0.3–0.4)*     |
| 20:3(n-9)            | 0.0 (0.0–0.0)    | 9.0 (2.6–13.8)*    | 0.0 (0.0–0.0)    | 6.3 (2.1–9.6)**    |
| 22:0                 | 1.1 (0.9–1.1)    | 1.0 (0.9–1.9)      | 1.4 (1.2–1.5)    | 1.1 (1.0–1.4)      |
| 20:4(n-6)            | 12.0 (9.4–14.4)  | 8.5 (4.3–11.3)*    | 12.4 (11.3–13.4) | 12.1 (7.2–15.7)    |
| 24:0                 | 1.1 (1.0–1.1)    | 0.7 (0.7–0.8)**    | 0.8 (0.8–0.9)    | 0.7 (0.7–0.8)      |
| 24:1(n-9)            | 0.5 (0.3–0.6)    | 0.9 (0.8–1.0)**    | 0.4 (0.3–0.4)    | 0.8 (0.6–1.0)**    |
| 22:6(n-6)            | 2.8 (1.7–3.9)    | 2.6 (1.6–2.9)      | 1.8 (1.7–2.0)    | 2.5 (2.1–2.8)**    |
| n-6/n-3              | 13.9 (10.5–19.3) | 6.5 (4.6–9.9)*     | 20.3 (19.0–21.6) | 8.7 (5.9–11.6)     |
| 18:1(n-9)/18:2(n-6)  | 0.1 (0.1–0.1)    | 2.1 (1.3–3.0)**    | 0.1 (0.1–0.1)    | 1.4 (0.9–2.0)**    |
| 20:3(n-9)/20:4(n-6)  | 0.0 (0.0–0.0)    | 1.1 (0.2–3.1)*     | 0.0 (0.0–0.0)    | 0.5 (0.1–1.3)**    |

Values are median (range).

Results with \* are \*\* significantly different in the neonatal rats from the dams receiving the EFA-S diet,  $p < 0.05$  and  $< 0.01$ , respectively compared with the neonatal rats from the dams receiving the EFA-D diet.

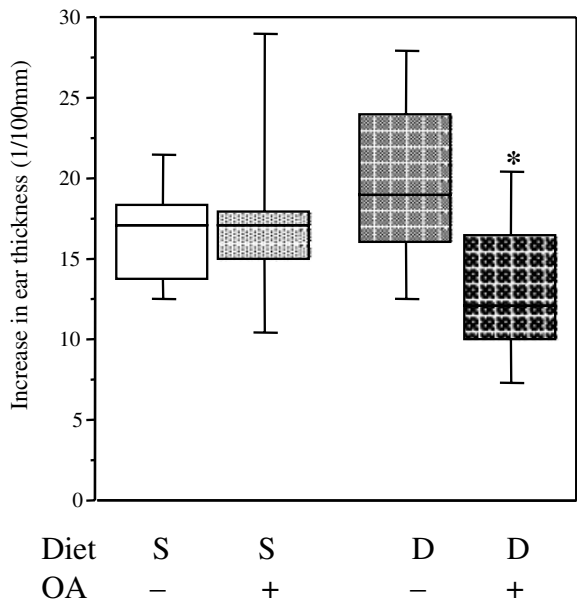


Fig. 5. The delayed-type hypersensitivity (DTH) reaction against ovalbumin (OA) in the offspring of dams fed the diet supplemented (S) with or deficient (D) in essential fatty acids and exposed to OA orally (+), or not exposed (–) before immunization. The results are expressed as box plots that indicate median value of nine 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.

In the PLNs, IL-4, IFN- $\gamma$  and TGF- $\beta$  mRNA expression were detected by RT-PCR at 7 days after booster immunization. The

TGF- $\beta$  mRNA levels were significantly higher in the PLNs from the offspring of the dams fed the D diet and provided with OA orally during lactation (Fig. 7). There was no difference in the mRNA levels of IL-4 or IFN- $\gamma$  in the PLNs among any of the groups of offspring (data not shown).

## Discussion

The present study shows that dietary intake of EFA had no effect on the induction of oral tolerance in adult rats, but influenced the development of tolerance in neonatal rats to a food antigen fed to their dams during lactation.

In adult rats after oral exposure to OA, an antigen-specific suppression of the DTH response to OA was observed in both the EFA-S and -D groups, accompanied by a reduction of DTH to an unrelated antigen HSA, an effect known as bystander suppression (21). In addition, in both the dietary groups of adult rats given OA orally the IgG antibody response to OA, as well as to HSA, was significantly reduced. Occurrence of bystander suppression is associated with the presence of regulatory T-cells that are triggered by a specific antigen and responsible for the release of the antigen non-specific suppressive cytokine TGF- $\beta$  (13). As a result, immune responses to other antigens in the close vicinity are also diminished.

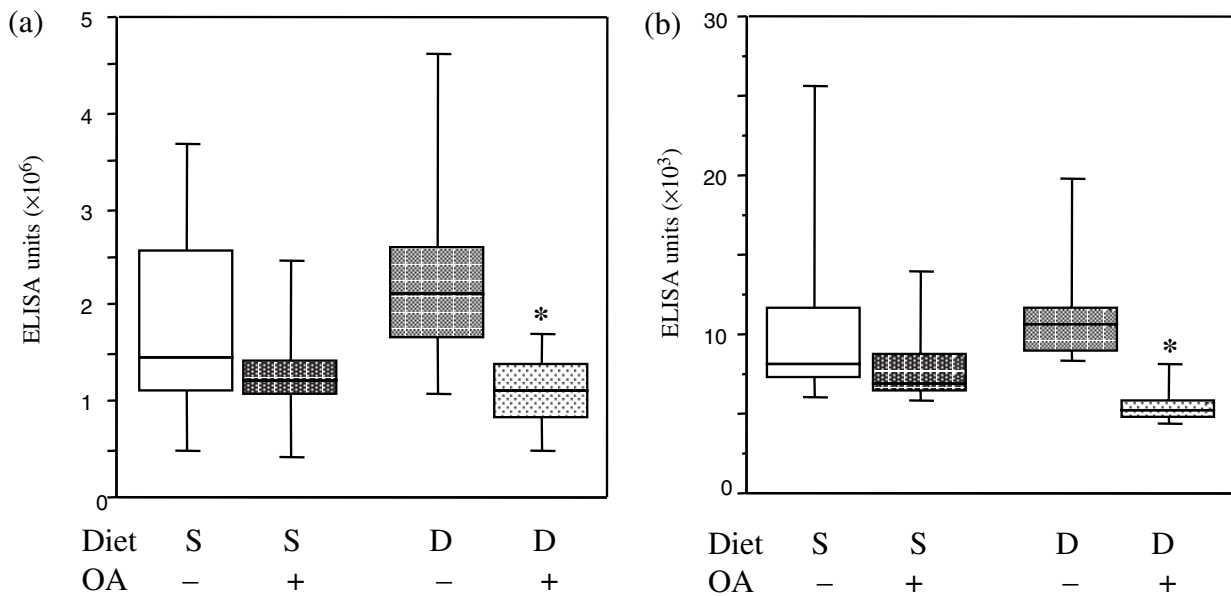


Fig. 6. Serum levels of IgG (a) and IgM (b) anti-ovalbumin (OA) antibodies in offspring from dams fed the diet supplemented (S) with or deficient (D) in essential fatty acids and exposed to OA orally (+), or not exposed (-) before immunization. The results are expressed as box plots that indicate median value of nine 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.

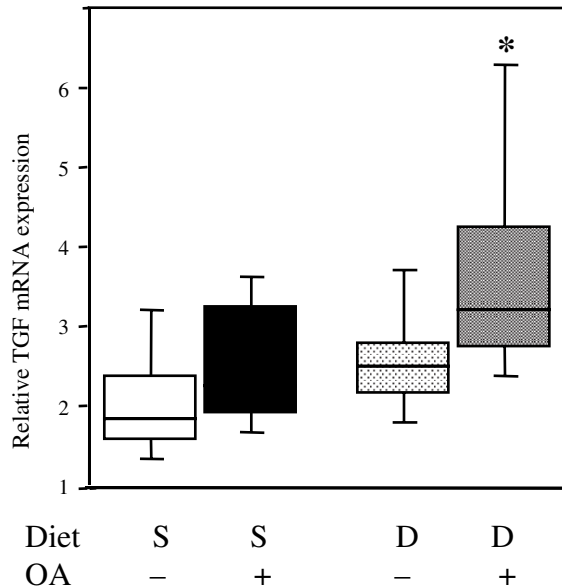


Fig. 7. Expression of transforming growth factor beta (TGF- $\beta$ ) mRNA in the popliteal lymph nodes from offspring of dams fed the diet supplemented (S) with or deficient (D) in essential fatty acids and exposed to OA orally (+), or not exposed (-) before immunization. The results are expressed as box plots that indicate median value of eight 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.

Feeding adult rats the D diet during 7 wk resulted in considerable changes in the FA composition of serum PL and, as we have shown earlier, in the FA composition of the intestinal mucosa PL (22). In addition, EFA deficiency influenced the ratio of n-6/n-3 EFA in serum PL, which was higher in adult rats on the S diet compared with the D group. Variations in the PL FA composition have been shown to affect the intestinal morphology and nutrient transport (23), as well as the eicosanoid production in the small intestine (24). Despite these differences the oral tolerance was maintained and mediated at least partly by an active suppression mechanism in the adult animals of both the dietary groups.

In contrast to adults induction of tolerance in the neonatal rats was affected by dietary EFA. In the offspring of the dams fed the D diet antigen exposure via the milk resulted in suppression of the DTH reaction to OA and suppressed levels of anti-OA antibodies, confirming the induction of tolerance. Previous results from our laboratory show that in rats made orally tolerant at adult age TGF- $\beta$  producing cells appeared in the draining PLN after immunization and presumably suppressed the PLN swelling seen during the immune response (21). In agreement with these data we found now that after the booster immunization with OA the draining PLN were less enlarged and the TGF- $\beta$  mRNA levels were significantly higher in the PLN of the offspring of

the dams fed the D diet and orally treated with OA. Thus, in the offspring of the dams fed the D diet immunological tolerance to a food antigen was induced and mediated at least partly by active suppression. In contrast, in the offspring of the dams fed the S diet this effect was not observed. The absence of suppression of the PLN swelling, as well as lack of differences in the TGF- $\beta$  mRNA seen in the offspring of the dams fed the S diet and exposed to OA indicated an abrogated development of regulatory T cells resulting in an inability to suppress the immune responses against OA. The results show that the diet D in EFA, rather than the one S with EFA, favoured the induction of oral tolerance in the neonatal rats via their mothers. Interestingly, markedly higher ratio of n-6/n-3 EFA in serum PL was detected in the offspring from the dams fed the S diet, which did not develop oral tolerance to OA. We assume that the balance between n-6 and n-3 EFA in the diet might be one of the factors important for the induction or failure of oral tolerance.

In the neonatal period the immune responses to dietary antigens may be regulated by antigen transmission via breast milk. The extent and/or mode of antigen exposure and antigen processing by both the mother and their offspring can influence this process, which is dependent on the functionality of the intestinal barrier, and the maturation stage of the immune system. Orally fed proteins are able to cross the gut mucosa of lactating rats and can be found in the serum and the milk (25). It has been shown that the FA composition of the mammary epithelial cell membranes affects synthesis, secretion, as well as intracellular transport of proteins in lactating mammary epithelial cells (26). This may influence both the quantity and quality of food antigens in the milk.

Another important factor required for appropriate antigen exposure of neonatal rats is the functionality of the intestinal barrier. Recently it has been shown that polyunsaturated FAs alter the intestinal barrier function reducing transcellular non-receptor-mediated permeation of proteins across a monolayer of the intestinal epithelium *in vitro* (27). We observed that the maternal dietary intake of EFA affected the PL FA composition of the jejunal mucosa of the rat offspring and might result in an altered protein transport across the enterocytes. Thus, failure to induce oral tolerance in the offspring of the mothers receiving the S diet might be due to inappropriate exposure to OA resulting from the altered antigen transfer to the milk and/or into the intestine.

It has been shown that in cultured intestinal cells protein transport and processing (28) is stimulated by IFN- $\gamma$ , thus increasing the antigenic load in the intestinal mucosa. The number of CD8-positive intraepithelial lymphocytes (IEL), known to produce IFN- $\gamma$  (29), in the jejunal villi was higher in the neonatal rats fed the D diet than in those fed the S diet. Decreased number of CD8-positive IEL might result in reduced IFN- $\gamma$  production and less efficient antigen transport into the intestinal mucosa.

The neonate's immature immune system might be especially sensitive to changes in local eicosanoid production caused by varying consumption of EFA and/or by n-6/n-3 EFA ratios. The main arachidonic acid metabolite, PGE<sub>2</sub>, is a potent modulator of dendritic cell (DC) functions (30), critical for the induction of primary immune response and immunological tolerance. The possible effects of EFA on production of PGE<sub>2</sub> and DC maturation need further investigation.

Our data indicate that the levels of EFA, linoleic 18:2(n-6) and  $\alpha$ -linolenic 18:3(n-3) acids in the maternal diet may have significant effects on the development of immunological tolerance to dietary antigen in their offspring. The results might have an impact on the understanding of the increased prevalence of atopic diseases in developed countries, possibly related to an increased n-6 EFA consumption in the Western diet (9, 10). Possible effects of n-6/n-3 ratios in maternal diet on the induction of neonatal tolerance need to be investigated.

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