Research Article

Role of vitamin A elimination or supplementation diets during postnatal development on the allergic sensitisation in mice

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Vitamin A (VA) and its derivatives, the retinoids, are important factors for the development of the immune system. It has been shown in adult animals that proliferation of lymphocyte populations and antibody secretion are retinoid dependent, while little is known about the effects of retinoids during postnatal development. The aim of this study was to investigate the role of VA on allergic sensitisation during lactation and after weaning using an *in vivo* system for postnatal allergic sensitisation in mice. Different VA diets (basal/VA elimination/VA (as retinyl palmitate) supplemented) were fed to the dams throughout lactation and directly to the pups after weaning. Allergic sensitisation was induced with a single peritoneal ovalbumin (OVA) injection at day 28 after weaning. The phenotype of lymphocytes was analysed by flow cytometry and functional data were obtained by analysis of (IL-4/IFN-γ) cytokine production and antibody production (OVA-specific IgG1 and IgE) in the offspring. VA/retinyl palmitate supplementation during lactation and after weaning decreased CD3+, CD4+, CD8+ and B220+ populations in splenic lymphocytes but also significantly enhanced IL-4 production and OVA-specific IgE after sensitisation. In contrast, mice fed VA-elimination diet displayed no significant alteration of lymphocyte numbers and a slightly increased IL-4 production. Our results showed that a single allergen injection during postnatal development induces allergic sensitisation whose degree is modified by the VA content of the maternal diet during lactation and the diet of the pups after weaning, indicating an important role of VA on the severity of the allergic sensitisation.

Keywords: Allergic sensitisation / Allergy / Atopic disease / Postnatal development / Retinoic acid

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1 Introduction

The development of the human and murine immune system shows similarities regarding T-cell development. Due to these and because of the limitations on studying human neonates murine studies are useful to understand the immune response during neonatal development. Nevertheless, major differences are observed in the kinetics of T-cell development between humans and mice. Differentiation of haematopoietic stem cells as well as clonal selection takes place before birth in human and mice. In contrast, lymphocyte proliferation and expansion occur in humans before birth and in mice mainly after birth (reviewed in [1]).

Allergic sensitisation in humans occurs mostly during the first year of life, indicating that processes like T-cell dif-

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Abbreviations: OVA, ovalbumin; **PE,** phycoerythrin; **RAR,** retinoic acid receptor; **VA,** vitamin A

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ferentiation and lymphocyte maturation are susceptible towards allergic sensitisation [2 – 4]. Only few studies have focused on the regulation of allergic sensitisation during postnatal development by using murine systems for allergic sensitisation [(5 – 9].

Allergic sensitisation is characterised by an abnormal balance in the Th1/Th2 responses. The shift in the normal Th1/Th2 balance towards a sensitisation state in newborns is influenced by various factors (reviewed in ref. [7, 10]). Among these factors, maternal (*in utero*) environment [7, 8, 11, 12], early bacterial or viral inflammation [13 – 15]. Other important factors are also dietary substances ingested by the mother during lactation and by the pup after weaning. While digested proteins may act as relevant food allergens (reviewed in ref. [16]), lipids like fatty acids, carotenoids and vitamins influence Th1/Th2 responses [17 – 20].

Several human studies suggest differences in fatty acid intake as an important factor for the onset of allergic diseases (reviewed in ref. [19, 21]), while limited reports postulate carotenoids and VA as possible skewing factors [22 – 24]. In previous studies by our group [22, 24] and others [20, 25 – 29], it has been shown that retinoids favour Th2 immune responses. In addition to modulation of the Th1/Th2 responses, retinoids are also involved in clonal T-cell selection and apoptosis [30, 31]. Respectively, apoptosis has also recently been shown to be altered in atopic diseases [32 – 34].

Vitamin A (VA) and its derivatives, the retinoids, have different activity mechanisms, which are relevant for the immune response. The most important is that retinoic acid in its all-*trans* or 9-*cis*-configuration act as highly potent activators of the retinoic acid receptors (RARs) and the retinoid-X-receptors (RXR). *Via* activation of these nuclear receptor pathways retinoic acid can directly influence gene transcription of various retinoic acid response genes [35].

We and other groups have shown that VA transfer as well as carotenoid transfer *via* mothers' milk is mainly influenced by maternal dietary VA/carotenoid intake in humans and in experimental animal models [36 – 39]. The Western diet has been associated with an increased incidence of allergic sensitisation. Possible reasons are that the Western diet is characterised by a high dietary intake of fat (especially n-6 PUFAs) as well as a high intake of retinoids [40, 41]. In addition, high dietary intake of fat both in humans and animal models has been associated with improved bioavailability of carotenoids in the diet, leading to elevated carotenoid and retinoid levels in serum and organs (reviewed in ref. [42, 43]).

The aim of our study was to investigate whether nutritional relevant retinoids affect allergic sensitisation during lactation and after weaning in a mouse allergy model. We studied the effects of a VA-supplementation or -elimination diet on serum and liver retinoid levels, relative distribution of lymphocyte subpopulations, cytokine secretion as well as allergen-specific antibody response within the different diet groups. Our hypothesis is that VA content of the diet influences the severity of allergic sensitisation already during postnatal period.

2 Materials and methods

2.1 Experimental animals and diets

The animal experiments were performed in the facilities of the Max Rubner Laboratory of the German Institute of Human Nutrition (DIFE) in Nuthetal, Germany. The respective ethical authorities for animal protection from the Land Brandenburg approved the experiments.

Balb/c mice (*Mus musculus*) were obtained from Tierzucht Schönwalde (Germany). The animals were kept under controlled conditions at room temperature (21 ± 1°C), a relative humidity of 55 ± 5% and 12-h light/dark cycle with light between 8:00 AM and 8:00 PM. The animals were mated between 6:00 PM until 7:00 AM of the following day. After detection of a vaginal plug, the pregnant mice were separated and set in individual cages. On day 21 after birth, the pups were separated from the mother and set in different cages separated by gender. Each group depending on ovalbumin (OVA) sensitisation or diet were minimum six and maximum ten pups, depending on the litter size.

The dams and pups were fed according to the schedule in Table 1/Fig. 1. Food and water were administered *ad libitum*. Mineral mix (Mineral-Spurenelemente-Vormischung C1000) and vitamin mix (Vitamin-Vormischung C1000 mix) for diet preparation were purchased from Altromin (Lage, Germany). The VA content of the diet is shown in Table 1 and no detectable amounts of carotenoids are reported in the vitamin mix.

The dams were randomly assigned to a specific diet and began to receive it 1 day before giving birth and until day

Table 1. General diet composition

Wheat starch	64%
Casein	12%
Sucrose	10%
Plant oil	5%
Mineral mix	5%
Vitamin mix	2%
Cellulose	2%
Content of VA in the diet (*per* kg diet)	
B	4.5 mg (4500 RE[a)])
VA-E	0 mg of VA (using a VA-free 'vitamin mix')
VA-S	122 000 RE as retinyl palmitate[b)] (216 mg of retinyl palmitate/kg diet supplemented)

a) RE – Retinol equivalents.
b) VA was administered as retinyl palmitate purchased from Sigma Chemical, Taufkirchen, Germany.
B, Basal diet; VA-E, VA-elimination diet; VA-S, VA-supplemented diet.

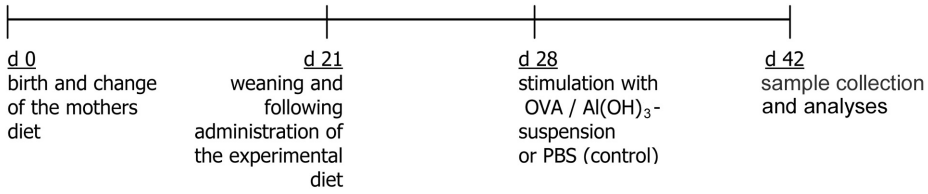

Figure 1. Experimental design of the study.

21 after birth (the day of birth was considered as day 0). The pups received the same diet as their mothers after weaning.

The pups were sensitised with 100 μL of OVA suspension (with 10 μg of OVA coupled to 1.5 mg of $Al(OH)_3$; Fig. 1) by a single intraperitoneal injection at day 28 after birth. Control animals received 100 μL PBS injection only. The pups were sensitised and investigated independently from their gender.

2.2 Sample collection

On day 42 after birth, the pups were anaesthetised under ether and blood was taken from the retro bulbous vein. In addition, liver and spleen were collected from the individual mice. Blood was centrifuged at $1300 \times g$ for 3 min to obtain serum and kept at $-20^{\circ}C$ until analysis. Liver was collected and frozen at $-80^{\circ}C$ until analysis. Spleen was collected, kept on ice and immediately prepared to obtain lymphocytes.

2.3 Cell preparation

For cell analysis and cell culture experiments, the spleens were prepared into single cell suspensions like previously described in Garcia *et al*. [44]. Shortly, splenocytes were harvested from the spleen by gently pressing the tissue through a fine Falcon® nylon mesh sieve (100 μm) (BD PharMingen, San Diego, CA, USA) and centrifuged at 1200 rpm at 4°C. In the second washing step, the cells were passed through 40 μM nylon mesh sieve and further centrifuged. The yielded cell pellet was reconstituted in 25 mL of PBS.

2.4 Flow cytometric analyses

Splenocytes (1×10^6 cells) were stained with mAb. The cell suspensions were mixed with 100 μL of labelled mAb and buffer (10% BSA in PBS) in a 1:200 dilution. A double colour staining using FITC and phycoerythrin (PE) was performed using the following mAb combinations: FITC-conjugated rat IgG_{2a}, with R-PE-conjugated rat IgG_{2a}; FITC-conjugated rat antimouse CD8a (Ly-2) with R-PE-conjugated rat antimouse CD4 (L3T4) and FITC-conjugated rat antimouse CD3 molecular complex with R-PE-conjugated rat antimouse CD45R/B220. All mAb were purchased from BD PharMingen. After 20 min at 4°C in darkness, the cells were washed. The supernatant was removed and the pellet resuspended with 500 μL of paraformaldehyde (2% in PBS) purchased from Sigma (Taufkirchen, D). The fixed cells were kept at 4°C until analysis. Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA, USA) with Cell quest 3.3 software.

2.5 Cytokine determinations

Splenocytes (10^6 cells) were cultured unstimulated or stimulated (RPMI medium containing 20 ng/mL PMA and 0.2 μM ionomycin) for 48 h. Supernatants were collected and kept frozen ($-80^{\circ}C$) until further analysis by ELISA. For detection of the cytokines (IL-4/IFN-γ) ELISA from R&D (Wiesbaden, D) were used according to the manufacturer's instructions.

2.6 Ig analysis

OVA-specific IgE, IgG1 and IgG2a antibody levels were measured by ELISA, as previously described [45, 46].

2.7 HPLC for retinoid analysis

Liver was thawed, weighted and immediately frozen at $-80^{\circ}C$ until analysis. For analysis, the liver was kept on ice and homogenised manually in a Teflon Glas Potter in a 1:10 dilution with Millipore water. Higher dilutions (up to 1:10 000) were made for samples of VA-supplemented mice. Dilutions were necessary for the determinations of high retinoid concentrations due to a calibration limit of 2000 ng/mL for the used HPLC method. Serum and organ samples were prepared according to the method described by Rühl and Schweigert [47]. Shortly, the serum and liver homogenates were extracted with a three-fold volume of isopropanol followed by SPE, vortexed for 10 s, shaken for 3 min at 20°C and centrifuged at $3000 \times g$ during 6 min. After centrifugation, the supernatant (410 μL) was taken and transferred to brown HPLC vials. Retinoids were determined using an RP-HPLC method [47].

2.8 Statistics

The results of the lymphocyte percentages, cytokine concentrations, retinoid concentrations and antibody levels are expressed as means with standard errors. The statistical analysis was performed using the SPSS 10.0 (SPBS, Chi-

Table 2. Retinyl palmitate and retinol concentrations in the serum and liver of the pups after weaning.

Diet	Stimulation	ROH in the serum (μg/mL)	RP in the serum (μg/mL)	ROH in the liver (μg/g)	RP in the liver (μg/g)
B	PBS	0.17 ± 0.05	0.031 ± 0.039	22.32 ± 11.61	175.98 ± 31.49
	OVA	0.19 ± 0.02	0.030 ± 0.014	14.23 ± 7.24	182.38 ± 39.22
VA-E	PBS	0.10 ± 0.01[a)]	UDL[a)]	0.32 ± 0.02[a)]	4.58 ± 0.21[a)]
	OVA	0.13 ± 0.01[b)]	UDL[b)]	0.45 ± 0.13[b)]	3.68 ± 1.37[b)]
VA-S	PBS	0.32 ± 0.04[a)]	1.32 ± 0.39[a)]	42.28 ± 6.53[a)]	3299.00 ± 288.40[a)]
	OVA	0.31 ± 0.06[b)]	2.43 ± 0.28[b, c)]	51.84 ± 9.33[b)]	3575.66 ± 668.63[b)]

B, Basal diet; ROH, retinol; RP, retinyl palmitate; UDL, under the detection limit; VA-E, VA-elimination diet; VA-S, VA-supplementation diet.
All concentrations were calculated based on $n = 6$ mice *per* group analysed. Pups have been treated on day 28 with 10 μg of OVA; control application with PBS.
a) ($p < 0.05$) in comparison basal diet/PBS at the same day.
b) Significant difference ($p < 0.05$) in comparison basal diet/OVA at the same day.
c) Significant difference ($p < 0.05$) in comparison to the control (PBS) at the same diet.

cago, IL, USA) software for Windows using the Mann Whitney test. A p value of 0.05 was used to determine statistical significance.

3 Results

3.1 Modulation of retinol and retinyl palmitate concentrations by different VA-containing diets

Serum retinol concentrations in the basal diet and VA-elimination diet were in the same range, while after VA supplementation significant increases were observed either in OVA or nonsensitised animals (Table 2). After VA supplementation, the serum retinol concentrations showed a twofold increase, while retinyl palmitate concentrations (1.32 ± 0.39 μg/mL in nonsensitised mice) increased about 40 times in comparison to the basal diet (0.031 ± 0.039 μg/mL in nonsensitised mice). There was no retinyl palmitate detectable in the mice fed VA-elimination diet (Table 2).

Retinol and retinyl palmitate concentrations in liver samples showed pronounced variations in comparison to the serum concentrations, due to homeostatic control of serum retinol levels by the retinol-binding protein. Liver retinol and retinyl palmitate concentrations were higher than serum concentrations because liver is the major storage organ for retinoids. As expected, if compared to the basal diet retinol and retinyl palmitate concentrations in the liver were lower after VA-elimination diet and higher after VA-supplementation diet (Table 1).

3.2 Effects of VA diets on lymphocyte phenotype depending on allergic sensitisation

The most pronounced difference between the diets was the decreased percentage of T-lymphocytes in VA-supplemented animals (18.7% CD3+ cells) in comparison to basal diet (38.1% CD3+ cells). In VA-supplemented animals,

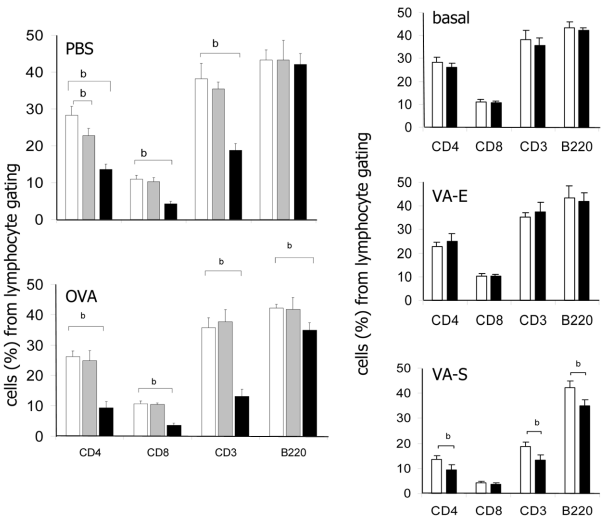

Figure 2. Distribution of lymphocyte populations in the spleen in different diet groups. The same data are presented in two sets of figures: (2.1.) summarised for the lymphocyte populations and (2.2.) for the different supplementation. (2.1.) Pups have been treated on day 28 with either 10 μg of OVA (Fig. 2.1., bottom) or 50 μL of PBS as control (Fig. 2.1., top). Basal diet (B) – open bars ($n = 9$ for PBS-treated animals and $n = 6$ for OVA-treated animals); VA-elimination diet (VA-E) – grey bars ($n = 10$ for PBS-treated animals and $n = 9$ for OVA-treated animals); VA-supplemented diet (VA-S) – black bars ($n = 6$ for PBS-treated animals and $n = 8$ for OVA-treated animals). (2.2.) Lymphocyte subpopulations after PBS treatment (open bars) or OVA sensitisation (black bars) after different supplementation as indicated in the figure. Significant differences are indicated by: b – ($p < 0.01$).

both CD4+ and CD8+ cell subpopulations were significantly downregulated (Fig. 2).

The VA-elimination diet in comparison to the basal diet decreased significantly the percentage of CD4+ cells from 28.3 to 22.8% (Fig. 2-1), while all other cell subsets were nonsignificantly decreased. In mice fed basal and VA-elimination diets, no significant alterations in the percentage of

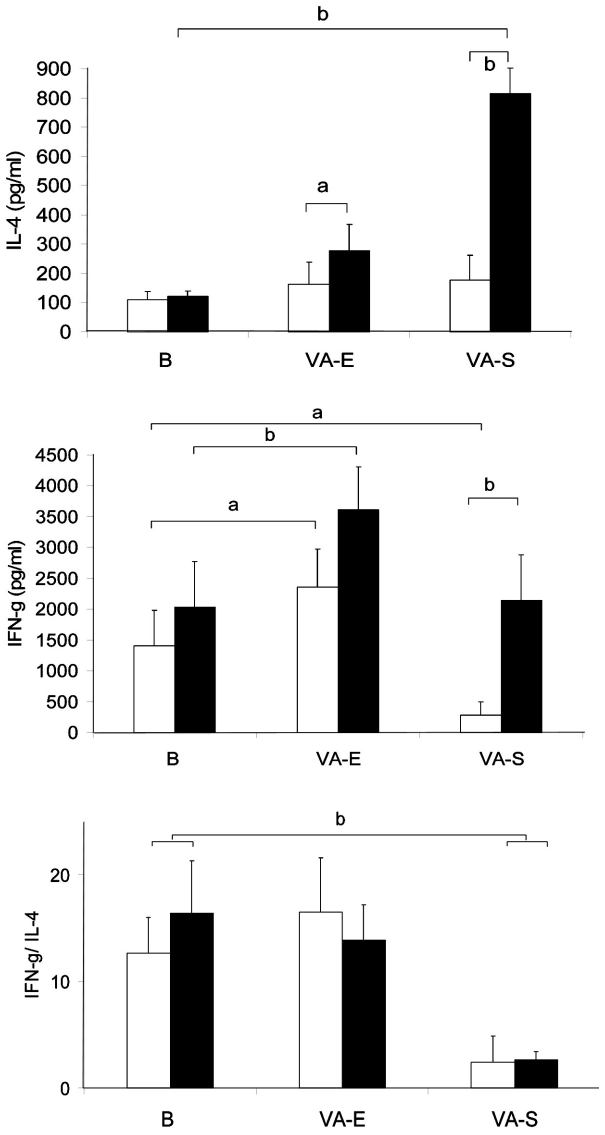

Figure 3. Effects of different diets on IL-4 (top figure) and IFN-γ secretion (middle figure) and IFN-γ/IL-4 ratio (bottom figure) of splenocytes after weaning. OVA-sensitised groups are shown as black bars and PBS-treated control as open bars. The figure is based on the following numbers of animals used basal diet ($n = 8$); VA-E diet ($n = 10$) and VA-S diet ($n = 6$) (for PBS-treated animals) as well as basal diet ($n = 6$); VA-E diet ($n = 6$) and VA-S diet ($n = 7$) (for OVA-treated animals). Splenocytes were previously stimulated with PMA/IONO. Cytokines were determined in supernatants. Significant differences are indicated by: a – ($p < 0.05$) and b – ($p < 0.01$). B, basal diet; VA-E, VA-elimination diet; VA-S, VA-supplemented diet.

lymphocyte subpopulations were observed for both PBS and OVA-sensitised animals (Fig. 2-2). On the other hand, in mice fed VA-supplemented diet after OVA sensitisation a significant reduction in the percentage of CD45R+/B220 cells (B-cells) was observed compared to the basal diet. In addition, a nonsignificant reduction was observed for CD3+ cells (T-cells) (Fig. 2-2). The percentage of B220+cells after OVA stimulation in mice was similar if fed the basal diet or the VA-elimination diet, but there were differences in the VA-supplemented diet. Mice fed VA-supplementation diet displayed decreased percentage distributions of B220+ cells, T-lymphocytes (CD3+) and their subpopulations (CD4+, CD8+) (Fig. 2-2).

VA supplementation in both sensitised and nonsensitised animals clearly decreased CD3+, CD4+, CD8+ and B220+ subpopulations. In contrast, VA elimination did not significantly alter lymphocyte percentages (Fig. 2-2).

3.3 Cytokine production after allergic sensitisation and dietary intervention

The IL-4 secretion in nonsensitised animals was independent from the animals' dietary treatment since no significant changes in IL-4 production between the diets were determined. After OVA-sensitisation IL-4 secretion was significantly enhanced in splenic cells from either VA elimination- and VA supplementation-diet fed mice, whereas IL-4 production remained unchanged in mice fed the basal diet (Fig. 3). In OVA-sensitised animals, a nonsignificant increase of IL-4 secretion could be observed in VA elimination-diet fed mice, while in VA-supplemented animals the increase was stronger and statistically significant.

The IFN-γ secretion in nonsensitised animals was strongly and significantly upregulated in VA elimination-diet fed mice and strongly and significantly decreased in VA-supplemented mice. After OVA-sensitisation IFN-γ secretion rose compared to nonsensitised animals independent from the diet, but reached significance exclusively after VA supplementation. IFN-γ secretion was significantly higher in OVA-sensitised mice receiving VA-elimination diet in comparison to mice fed basal diet.

The ratio of IFN-γ/IL-4 slightly changed after OVA sensitisation in each diet group. Between the basal and VA-elimination diet, the ratios were comparable. However, after VA supplementation the IFN-γ/IL-4 ratio decreased significantly from 12 – 16 to ~2.5 (Fig. 3).

3.4 Specific antibody response after allergic sensitisation and various retinoid-containing diets

As expected, OVA-specific IgG1 concentrations were not detectable (under the quantification limit) in all animals without OVA sensitisation independently from the supplemented diet. IgG1 concentrations after OVA sensitisation in animals fed the basal diet was 2.2 ± 1.6 µg/mL and increased after the other diets. A significant increase was only observed after VA supplementation with 17.7 ± 25.4 µg/mL (Fig. 4). In contrast, OVA-specific Ig2a levels were always below the detection limit in all examined animals (data not shown).

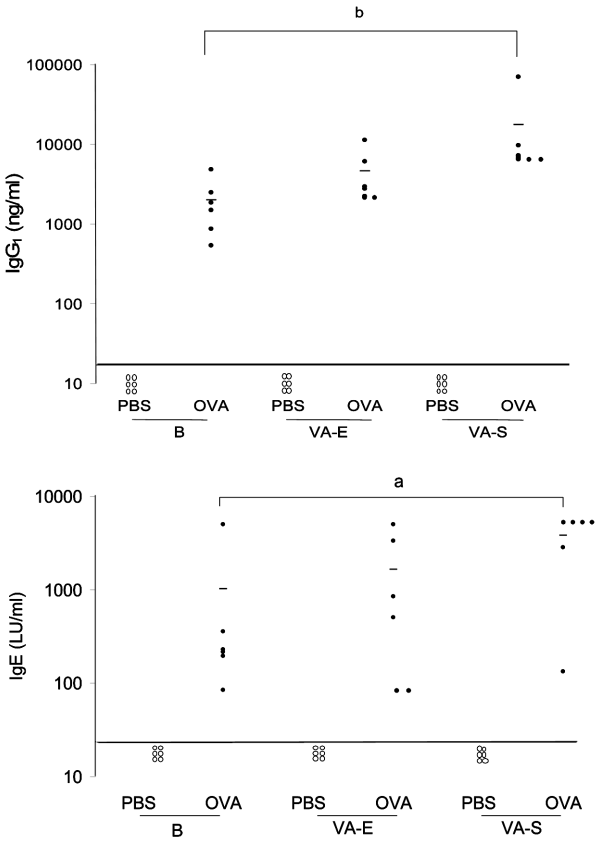

Figure 4. Concentrations of OVA-specific antibodies in serum after weaning. Top figure: specific IgG1 concentrations in ng/mL; bottom figure: specific IgE in LU/mL. Displayed are the single values ($n = 6$; for IgG1/$n = 6$ for IgE) as black dots (•) and the mean as a black bar. Values below the detection limits are shown by open symbols (○). Significant differences are indicated by: a – ($p < 0.05$) and b – ($p < 0.01$). B, basal diet; VA-E, VA-elimination diet; VA-S, VA-supplemented diet. The horizontal line in the figures marks the detection limit of the used method, while the maximum range is 5000 LU/mL.

OVA-specific IgE concentrations could not be detected in non-OVA-sensitised animals independent from the diet, as expected. In OVA-sensitised animals fed the basal diet, IgE concentrations were 1015 ± 1784 laboratory units (LU)/mL. VA supplementation resulted in significant higher levels of OVA-specific IgE (3827 ± 1832 LU), whereas in the VA-elimination diet IgE (1642 ± 1832 LU) concentrations showed nonsignificant changes.

4 Discussion

In the present study, we demonstrated that the VA content of the diet ingested by the mother during lactation and ingested after weaning by the pups strongly influenced the outcome of an allergic sensitisation.

In mice, early sensitisation at day 28 after birth results in an allergic sensitisation comparable in regards of quality to adult mice (reference to our data [48]). In this early phase of life, it is not possible to perform the usual triple OVA-sensitisation protocol over a period of 4 wk, this is the main reason why the severity/quantity of this single OVA-stimulated sensitisation is not comparable. In OVA-sensitised mice receiving basal diet OVA-specific antibodies of IgG1 and IgE class, which are markers of a Th-2 phenotype [49], were detected, whereas the Th-1-specific antibody IgG2a could not be detected. IFN-γ, a Th-1 cytokine, was slightly upregulated after OVA sensitisation, but surprisingly no increase in the secretion of IL-4, a Th-2 cytokine, was observed. Our study on early sensitisation using only a single injection of an allergen clearly demonstrates that the postnatal period is a sensitive period to induce allergic sensitisation. Due to the fact that lymphocyte proliferation and differentiation mainly takes place during postnatal development the presence of allergens during this life-phase may increase the susceptibility for allergic sensitisation. Additional experiments for the induction of allergic sensitisation in mice using a single OVA injection at day 7 after birth resulted in an increased production of IL-4 at day 21 after birth; however, OVA-specific IgE and IgG1 antibodies could not be detected (unpublished data).

An important outcome of our experiments is that the content of the VA in the diet strongly influences the result and intensity of an immune response after early postnatal allergen exposure. Three different diets have been given to the animals, either containing physiological and high non-nutritional relevant VA concentrations: VA basal diet, VA-supplementation diet and VA-elimination diet. First, retinoid levels found in mice fed basal diet are comparable to other plasma retinol concentrations reported by our group in neonatal NMRI-mice, while due to retinol homeostasis the levels are also comparable to adult NMRI [50] or Balb/c [18] mice. Retinyl palmitate, a representative retinyl ester, was found in low concentrations in serum, an expected result due to VA-homeostatic regulation.

As reported by us and other groups, an upregulation of retinol and retinyl palmitate levels in liver was observed after VA supplementation and a downregulation after VA-elimination diet. Comparable to a previous study, retinol concentrations in VA-supplemented animals nearly doubled [51]. VA elimination-diet fed mice displayed low serum VA levels and even decreased concentrations of VA in the liver. This is due to low VA levels of the pups in the early days of life and low VA transfer *via* mothers' milk as described by Green *et al*. [52] and Davila *et al*. [38]. Retinoid concentrations of OVA-sensitised or nonsensitised animals showed marginal differences, this may be explained by increased VA consumption during allergen challenge [53]. Only in VA supplemented-diet fed animals, a significant increase of liver retinol and a nonsignificant increase of serum and liver retinyl palmitate concentrations were determined.

The immunological analysis of sensitised mice receiving different VA-containing diets clearly shows that various

parameters including the percentage amounts of lymphocytes, cytokine secretion and specific serum antibody levels are significantly altered. Comparable to our initial study, where we extensively examined lymphocyte populations in the spleen in nonsensitised animals during postnatal development [44], a reduction of CD3+, CD4+ and CD8+ populations was observed after VA-supplementation diet which is possible due to increased apoptosis of lymphocytes by retinoic acid [31].

VA-elimination diet upregulated basal IFN-γ secretion in nonsensitised mice and in OVA-sensitised mice. Alteration and especially upregulation of IFN-γ secretion in animals on VA-elimination diet has been reported previously by several groups [54 – 56]. Our experiments first describe this upregulation following single allergen stimulation in animals at very young age. These results indicate that postnatal development is a susceptible period for allergic sensitisation. Surprisingly, due to higher IFN-γ and just slightly enhanced IL-4 secretion in mice there was no decrease of OVA-specific antibody secretion and even higher IgG1/IgE production. We speculate that other mechanisms rather than just simply IL-4 driving IgG1/IgE production may be involved. The involvement of other pathways resulting in higher IgG1/IgE production has been mentioned in reviews by Stokes and Casale [57] and Savelkoul and van Ommen [58]. Further research of our groups will focus on the involved mechanisms.

The VA supplementation led to reduced lymphocyte percentages, increased IL-4 secretion in sensitised animals, reduced IFN-γ secretion in nonsensitised animals and finally resulted in a strong increase of OVA-specific antibody production. These effects were mainly accounted to the active VA derivative all-*trans*-retinoic acid (ATRA), a potent activator of the nuclear retinoic acid receptor. An activation of RAR-response pathways has been reported to be associated with a Th1 to Th2 switch and further induction of allergic sensitisation pathways [22, 24 – 26]. Previous studies reported regulatory feedback mechanisms in mice for maintaining retinoic acid concentrations during VA deficiency [59, 60]. This may explain, why after VA-elimination diet in comparison to the basal diet the IgG1 and IgE levels were comparable. A novel study by Li *et al*. [61] described a thymic stromal lymphopoietin (TSLP) dependent allergic sensitisation in mice using RAR and/or vitamin D receptor (VDR) agonists for initiation of allergic dermatitis.

Regarding the data obtained after VA supplementation, extrapolation of our data to humans or pet animals is difficult or even not possible, because the dose used for the dietary supplementation is not nutritionally relevant and not directly applicable to humans. However, no toxic effects were observed in dams and newborns after each individual diet, which is in accordance to previous publications by our group [39, 51]. Comparable to our studies [39, 51], the VA-supplementation diet contained high concentrations of VA, which resulted in great increases of retinol concentrations in the liver as well as retinyl palmitate concentrations in serum and liver. Remarkable are the increased retinyl palmitate concentrations in the serum of the pups after VA-supplementation diet, in adult animals these increased concentrations of retinyl ester have just been reported when using toxic VA doses [62]. This suggests that younger animals with low initial VA sources could be more susceptible to higher concentrations of VA *via* mother milk [39].

The VA-elimination diet could explain a nutritional relevant situation often occurring in third world and developing countries, but in these countries allergies mainly exclusively a health problem observed in population with adopting ‚Western style' diet [63]. However, previous epidemiological studies in Western countries have not clearly pointed out a direct association between VA-intake and type-1 sensitisation. In epidemiological studies, high intake of pro-VA carotenoid β-carotene correlate with an increased risk of hay fever [64], while low intakes of α-carotene are associated with an increased risk in asthma [65]. Serum concentrations of the pro-VA carotenoids α-carotene and β-cryptoxanthin were inversely associated with allergic skin sensitisation [66], while α-carotene levels tended to be associated with increased risk of allergic sensitisation [67]. Novel studies by our labs described increased concentrations of ATRA [68] after 2-wk carrot juice supplementation (high in β-carotene) and an increased secretion of the Th-2 cytokine IL-4 in the same group [17]. In addition, there is a tendency towards an increase in concentrations of pro-VA carotenoids were reported in groups with a higher incidence of allergic sensitisation [69]. Until date, it is not clear whether VA or pro-VA carotenoids obtain an influence on the rate and severity of allergic sensitisation.

Our experiments in mice clearly show that allergic sensitisation is influenced by the VA content in the diet. Further studies should focus on the human nutritional status and its clinical relevance by analysing the interaction of retinoid concentrations, retinoid-binding proteins, retinoid-metabolising enzymes and retinoid receptors which are influenced in the human organism and thereby could influence allergic sensitisation.

In summary, our experiments show that a single exposure with an allergen during postnatal development induces an allergic sensitisation, which is increased after VA-supplementation diet.

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Addendum

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