

Vitamin A Deficiency Decreases and High Dietary Vitamin A Increases Disease Severity in the Mouse Model of Asthma¹

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The Th1/Th2 paradigm has become an important issue in the pathogenesis of asthma, characterized by normal Th1 and elevated Th2 cytokine expression. Vitamin A deficiency (VAD) can produce a Th1 bias, whereas high-level dietary vitamin A can promote a Th2 bias. We used the OVA exposure mouse model to determine the contributions of vitamin A-deficient, control (4IU/g), and high-level vitamin A (250-IU/g) diets to the development of allergic airway inflammation and hyperresponsiveness. VAD reduced serum IgE and IgG1 responses, pulmonary eosinophilia, and the levels of IL-4 and IL-5 in bronchoalveolar lavage specimens, whereas the 250-IU/g diet increased serum IgE. Also, VAD blocked pulmonary hyperresponsiveness following methacholine challenge while the 250-IU/g diet exacerbated pulmonary hyperresponsiveness. In conclusion, VAD diminished and high-level dietary vitamin A enhanced the development of experimental asthma in this model system. These data suggest that excessive intake of vitamin A may increase the risk or severity of asthma in industrialized countries whereas vitamin A deficiency continues to increase mortality from infectious diseases in developing countries. *The Journal of Immunology*, 2008, 180: 1834–1842.

The “hygiene hypothesis” postulates that the high burden of infection among infants and young children in developing countries biases the immune response against the development of atopic disease and asthma, perhaps by dampening Th2 responses to allergens. In contrast, children in developed countries have a lower burden of infection and this may predispose them to respond to allergens with a more prominent Th2 response. This “unnatural” Th2 bias may contribute to the higher prevalence of asthma in developed countries (1). However, the hygiene hypothesis does not consider the substantial dietary differences that exist between children in these countries. For example, vitamin A deficiency (VAD)³ is common in developing countries and contributes to an increased risk of death from common infectious diseases (2). Animal studies indicate that VAD produces a significant Th1 bias (3–5), although data are not completely consistent (6). In contrast, high-level dietary vitamin A produces a Th2 bias (7). In addition to dietary studies, genetic and pharmacological approaches also offer support for vitamin A promoting Th2 responses. The active metabolite of vitamin A, retinoic acid, acts to regulate gene transcription via two families of nuclear receptors, the retinoic acid receptor (RAR- α , - β , - γ) and the retinoid X receptor (RXR- α , - β , - γ) (8). In agreement with the data from dietary studies, disruption of the *Rxr α* gene can produce a Th1 bias (9, 10) and treatment of mice with a retinoid X receptor antagonist

can decrease Th2-mediated pulmonary eosinophilic inflammation (11).

Thus, one can postulate that the relatively high intake of vitamin A in infants and young children in industrialized countries may contribute to a Th2 bias and an increased risk of asthma. Data from human studies to support this diet hypothesis are scarce. However, a recent epidemiologic study in the US found that multivitamin supplementation (which would include vitamin A) within the first 6 mo of life was associated with an increased risk for asthma (12). In addition, the use of combined vitamin A and D supplements with high bioavailability in Sweden has also been associated with increased risk of asthma later in life (13). The authors postulated that these vitamins produced a Th2 bias.

The OVA-induced mouse model of allergic pulmonary inflammation and hyperresponsiveness is commonly used to examine basic immunologic mechanisms that contribute to the development of asthma (14). We have used this model system to test the hypotheses that VAD impairs the development of OVA-induced Ab responses (IgE and IgG1), pulmonary eosinophilia, Th2 cytokine production, and hyperresponsiveness relative to mice fed a control diet (4-IU of vitamin A per gram of diet). We also test the hypothesis that high-level dietary vitamin A (250-IU/g) would exacerbate these responses. In general, we found that VAD impairs and high-level dietary vitamin A enhances the development of experimental asthma in this model system.

Materials and Methods

Animals

All procedures were performed under a protocol approved by the University of California at Davis Institutional Animal Care and Use Committee (Davis, CA). Timed-pregnant female C57BL/6 mice were purchased from The Jackson Laboratory. The mice were housed in positive ventilated air-filtering system (TouchSLIMLine air handling unit; Tecniplast) facilities on a 12-h light, 12-h dark cycle. Mice were fed the diets ad libitum.

Diets

Beginning during week 2 of gestation, all pregnant females were fed a vitamin A-deficient diet (0 IU/g diet vitamin A in experiment 1 and 0.2 IU/g in experiment 2). After birth, the dams either stayed on the VAD diet or were placed on a control diet with the recommended vitamin A intake for mice (15) (4-IU/g) or on a diet with high vitamin A content (250-IU/g), as we have used previously (7). Mice remained on these same diets after

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Received for publication July 27, 2007. Accepted for publication November 16, 2007.

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¹ This work was supported by National Institutes of Health Grants R01AI50863, K08HL076415, and United States Department of Agriculture Current Research Information System Project 5306-51530-006-00D.

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³ Abbreviations used in this paper: VAD, vitamin A deficiency; AHR, airway hyperresponsiveness; AIR, sham aerosol; BAL, bronchoalveolar lavage; Mch, methacholine.

weaning. All diets were semipurified and casein-based. Diets were prepared and pelleted by Harlan-Teklad and follow the standard composition for growth and development (15). The casein and fat were stripped of fat-soluble nutrients to ensure they are free of additional vitamin A content.

Mice sensitization and challenge protocols

In both experiments the same experimental design was followed. Serum retinols were determined from two male and two female animals per diet group to assure that serum retinol levels were significantly lower in the VAD group as compared with the 4-IU control diet group before immunization. Immunization was begun at 7 wk of age in the first experiment and 6 wk in the second experiment. Mice were immunized via two i.p. injections containing both chicken egg OVA, (10 μ g; Grade V, Sigma-Aldrich) and with the carrier-hapten complex OVA-DNP (10 μ g; catalog no. D-5051-100 Biosearch Technologies), and alum (1 mg; Sigma-Aldrich) as an adjuvant in 0.2 ml of PBS on days 1 and 15 of the experiment prepared as described (16). Two to 3 wk after the second immunization, mice received 6 exposures to OVA aerosol, 10 ml of a 10 mg/ml (1%) solution. Mice were exposed 45–60 min until the complete OVA dose was administered. Mice were exposed three times per week as previously described (17). The last OVA exposure was given on the same day that mice were killed for assessment of pulmonary resistance and compliance and for tissue collection.

Pulmonary physiology

A subset of mice underwent methacholine (MCh) challenge using a whole body plethysmograph (Buxco) before tissue harvesting. During this procedure, dynamic compliance and resistance of the respiratory system were measured on deeply anesthetized, tracheotomized, and ventilated mice as previously described (18). Briefly, mice were deeply sedated and anesthetized with medetomidine (0.5 mg/kg; Dormitor, Western Medical Supply) and tiletamine/zolpidem (0.5 mg/kg; Telazol, Fort Dodge Laboratories). Mice were ventilated at 7–8 cm³/kg with a mouse ventilator (MiniVent; Hardward Apparatus). Total lung resistance and compliance were measured at baseline and immediately following a serial 3-min nebulization of saline and MCh (0.5–2.0 mg/ml). Total lung resistance and compliance parameters are computed for each breath from the flow and airway pressure signals throughout the respiratory cycle. The breath to breath calculations were then averaged over 3-min intervals and recorded. The software calculates the measurements are based on the equation $P = QR + V/C$ (P , pressure; Q , flow; R , resistance; V , volume; and C , compliance). All measurements were made in the same manner.

Whole lung lavage and tissue collection

Mice were euthanized with an overdose of pentobarbital and phenytoin (Buthanasia-D Special, Schering-Plough Animal Health) for the collection of bronchoalveolar lavage (BAL) fluid and tissues samples. BAL fluid was retrieved from each animal via cannulation of the exposed trachea and gentle flushing of the lungs with two 1-ml aliquots of PBS. Aliquots were pooled for individual animals preceding centrifugation (10 min, 650 $\times$ g, room temperature) for the separation of pelleted cells and supernatant. Supernatants were stored at -80°C for cytokine and chemokine analyses. Total counts of BAL cells were determined using a Cell-Dyne system (Abbott Diagnostics). Cytospin preparations of BAL cells and blood smears (experiment 2 only) were stained using Hema-3 differential stain (Fisher Scientific) to determine relative cell populations using standard morphological criteria. Blood was drawn by cardiac puncture and serum collected in K2 EDTA Vacutainer tubes (BD Pharmingen) for total IgE and DNP-specific IgG1 titer determinations. For histological evaluation, lungs were fixed at 30-cm pressure with 1% paraformaldehyde for 30 min and then immersed for at least 24 h and processed for light microscopy in paraffin, stained with H&E. Livers and lungs that were not used for histology were snap frozen in liquid nitrogen and stored at -80°C for later analyses.

Cytokine and chemokine analyses

Assessments of cytokine profiles from the BAL were performed using commercial Beadlyte mouse multicytokine flex kit reagents (Millipore) and a Bio-Plex suspension array system (Bio-Rad). Simultaneous measurement of IL-4, IL-5, and TNF- α was performed in the first experiment and that of IL-4, IL-5, TNF- α , and IP-10 in the second experiment. All assays were performed according to the manufacturer's protocols. Cytokine concentrations were determined using Bio-Plex software with four-parameter data analysis.

Serum IgE and IgG1 ELISA

Amounts of total IgE in serum were detected using the BD OptEIA ELISA kit (BD Pharmingen) following the company's instructions. For each mouse, duplicate sera samples were analyzed. Tetramethyl-benzidine substrate was used to develop the assay, which was read at 450 nm with correction at 570 nm by a PowerWave microplate reader (Bio-Tek Instruments).

To quantify DNP-specific Abs, serially diluted serum samples were added to 96-well flat-bottom plates coated with DNP-conjugated BSA (catalog no. NC9979122; Fisher Scientific) in PBS. After washing, bound Abs were detected using biotin-conjugated goat anti-mouse IgG1 (catalog no. M32115) from Caltag Laboratories followed by streptavidin-conjugated HRP (Amersham Biosciences) and a tetramethyl-benzidine substrate (BD Pharmingen). Absorbance was measured at 450 nm using an ELX 800 microplate reader (Bio-Tek Instruments). A standard curve was created using serial dilutions of a mouse serum with known concentrations of Ab isotypes (catalog no. 64901; MP Biomedicals) and wells precoated with goat anti-mouse Ig (catalog no. M30900; Caltag Laboratories).

Determining retinol levels in livers and lungs

Retinol from livers and lungs was measured by reverse-phase HPLC following saponification of retinyl esters (Waters Chromatography Division of Millipore) with small modifications as has been described (19, 20). Livers and lungs (10 to 50 mg) were dried by grinding in 100 mg of anhydrous sodium sulfate and saponified in 500 μ l of 6% ethanolic potassium hydroxide containing pyrogallol (1% w/w) for 1 h at 60°C in the dark. Retinol was extracted twice into 1 ml of hexane after resuspension in 500 μ l of water. The pooled and dried extracts were redissolved in 350 μ l of methanol and analyzed by HPLC (Waters Chromatography Division of Millipore) and a mobile phase of acetonitrile:isopropanol:methanol:ammonium acetate (63.75:25:11.25:0.000075; v/v). Retinol was detected at 325 nm on a Waters Photodiode Array Detector 996.

Statistical analysis

Statistical analysis was done with SigmaStat for Windows version 3.10 (Systat Software). Variables were assessed for normal distribution using the Kolmogorov-Smirnov test and transformed as needed. If transformation did not normalize the distribution, data were converted to ranks for analysis. Rank transformation was only needed for some comparisons of the pulmonary physiology data. Body weights were compared using two-way repeated measures ANOVA (the two variables being diet and age) for males and females separately, comparing the VAD and 250-IU groups to the 4-IU control group. Variables were compared at single time points (e.g., tissue vitamin A stores, serum IgE and IgG1, BAL cell counts, and cytokine concentrations) using two-way ANOVA comparing diet groups and sex. If means differed by sex or if a significant diet $\times$ sex interaction was seen (indicating a different diet effect in males vs females), then the analysis was repeated by one-way ANOVA in both sexes separately. All pairwise comparisons among diet groups were made using Duncan's multiple range test to adjust statistical significance for multiple comparisons. Airway physiology data were analyzed by two-way repeated-measures ANOVA (the two variables being diet and methacholine challenge dose) for the sham aerosol (AIR) and OVA aerosol treatment groups separately. In addition, two-way repeated measures ANOVA was also used to compare the AIR and OVA treatments within each diet group (see Table I). Data in the text are reported as mean $\pm$ SE.

Results

Effects of dietary vitamin A on body weight

In two independent experiments we investigated the effects of vitamin A status on the immune response and disease severity in the murine OVA model of allergic airway inflammation and hyperresponsiveness. VAD was produced with semipurified, casein-based diets containing either no vitamin A (0 IU/g diet; experiment 1) or 0.2 IU/g (experiment 2). VAD mice in experiment 1 reached a weight plateau (Fig. 1 and described below) that is indicative of severe deficiency; thus, we added a minimal level of vitamin A in experiment 2 to prevent the development of severe deficiency during the study period. In both experiments a control diet containing the recommended intake, 4-IU/g, and a high-level diet containing 250-IU/g were used.

In the first experiment, male mice on the VAD and 250-IU diets were significantly heavier than male mice on the control 4-IU diet

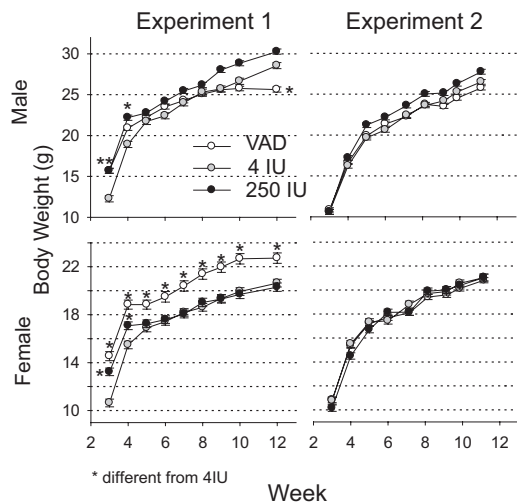


FIGURE 1. Growth curves of mice fed different dietary levels of vitamin A (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet). Results are shown in mean $\pm$ SE. An asterisk (*) indicates a significant difference from the 4-IU mean on the same day.

immediately after weaning at 3 and 4 wk of age, but these differences did not persist (Fig. 1). However, we observed a significant drop in the body weight of male VAD mice relative to the 4-IU control group at week 12, indicating that the male mice were severely deficient at the end of experiment 1. Throughout the first experiment female VAD mice were significantly heavier than control mice, apparently due to chance allocation of litters to the different diets at the beginning of the experiment. Female mice on the 250-IU diet were also heavier than the 4-IU mice at 3 and 4 wk of age, but this difference did not persist. No significant differences in body weight were seen throughout the second experiment.

Vitamin A stores in liver and lungs

Vitamin A levels in livers and lungs differed significantly among all dietary groups in both experiments (Fig. 2). In the first experiment there was a statistically significant diet $\times$ sex interaction; female VAD mice had significantly higher mean hepatic stores than VAD male mice (3.5 vs 1.5 nmol/g). The mean liver vitamin A levels for all mice in the VAD, 4-IU, and 250-IU groups in the first experiment were 3, 138, and 4,870 nmol/g, respectively. The corresponding levels in the second experiment were 3, 181, and 7,888 nmol/g, respectively. The mean lung vitamin A levels for all mice in the 4-IU and 250-IU groups in the first experiment were 110 and 537 nmol/g, respectively (VAD lungs were not analyzed). The mean lung levels in the VAD, 4-IU and 250-IU groups in the second experiment were 7, 54 and 1,744 nmol/g, respectively.

Vitamin A increases serum IgE and IgG1 responses

In the first experiment IgE was higher in females ($3,145 \pm 114$ ng/ml) than in males ($2,555 \pm 130$ ng/ml; $p = 0.032$), but no statistical interaction was seen between sex and diet. Mean IgE concentrations in the three diet groups all differed significantly from one another ($p < 0.001$ for all comparisons), with the IgE levels increasing as dietary vitamin A increased. The overall mean IgE concentrations were $1,694 \pm 174$, $3,070 \pm 143$, and $4,086 \pm 127$ ng/ml for the VAD, 4-IU, and 250-IU diets, respectively (Fig. 3). IgE levels were again higher in females than males in the second experiment, but the difference was not significant ($p = 0.10$) and no interactions was seen between diet and sex. The overall mean IgE concentrations were $2,126 \pm 177$, $2,016 \pm 157$, and

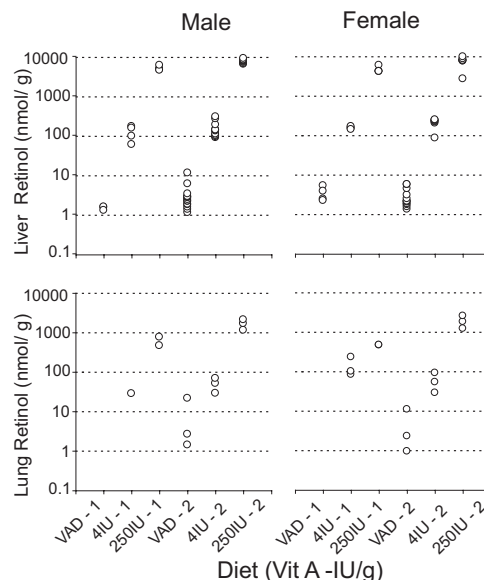


FIGURE 2. Vitamin A (Vit A) stores (nanomoles of retinol per gram of tissue) in liver and lungs from individual mice fed different levels of vitamin A (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet). Liver and lung stores differed significantly among all diet groups in both experiments. Lung vitamin A levels were not measured for VAD mice in experiment 1.

$2,860 \pm 210$ ng/ml for the VAD, 4-IU, and 250-IU diets, respectively, with the 250-IU mean being significantly greater than both the 4-IU ($p = 0.0035$) and the VAD ($p = 0.0064$) means, although the VAD and 4-IU means did not differ from one another ($p = 0.44$) (Fig. 3).

In the first experiment, DNP-specific IgG1 titers were higher in the 4-IU and 250-IU groups than in the VAD group (Fig. 3) and did not differ by sex. The overall means were 3.9 ± 1.8 , 8.0 ± 1.6 , and 13.1 ± 1.4 ng/ml for the VAD, 4-IU, and 250-IU groups, respectively. Both the 250-IU ($p = 0.002$) and the 4-IU ($p = 0.019$) means differed from the VAD mean but did not differ from one another ($p = 0.32$). In the second experiment, values were

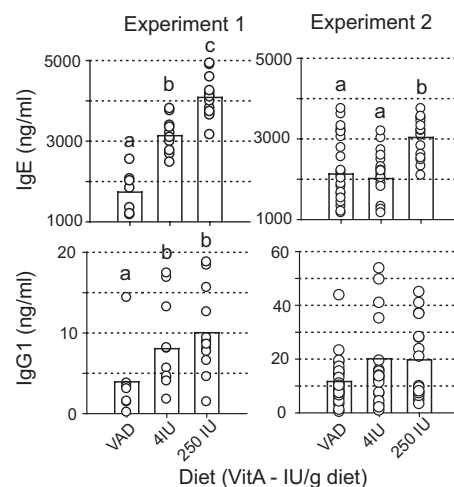


FIGURE 3. Total serum IgE and DNP-specific IgG1 responses in mice fed different levels of vitamin A (VitA) (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet). Within each figure different superscript letters indicate that group means are significantly different. Bars indicate means whereas data points represent results from individual mice.

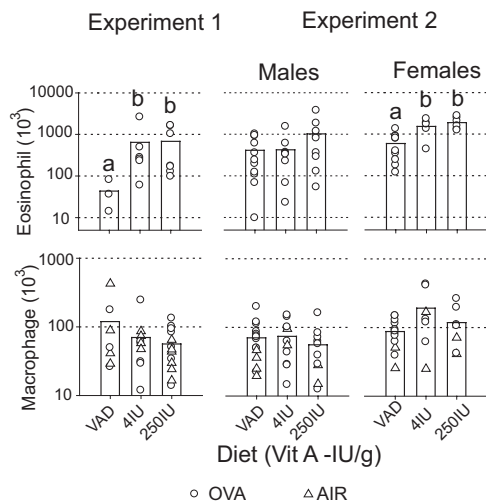


FIGURE 4. Bronchoalveolar lavage eosinophil and monocyte counts from mice fed different levels of vitamin A (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet). Within each figure different superscript letters indicate that group means are significantly different. Eosinophil data are from mice receiving aerosol treatment with OVA only. Macrophage data are from mice receiving both OVA and AIR, the sham aerosol treatment. Bars indicate means whereas data points represent results from individual mice.

higher in males than females ($p = 0.019$) but no difference was seen by diet ($p = 0.34$). The overall means were 14.1 ± 2.7 and 8.8 ± 1.7 ng/ml for male and female VAD mice, 20.2 ± 5.2 and 20.0 ± 5.3 ng/ml for the 4-IU mice, and 25.2 ± 4.8 and 12.7 ± 4.6 ng/ml for the 250-IU mice, respectively.

Vitamin A deficiency significantly decreases pulmonary eosinophilia

BAL eosinophils. VAD significantly diminished the development of pulmonary eosinophilia in mice treated with OVA aerosol. BAL eosinophilia did not develop in AIR-treated mice (data not shown). In experiment 1, eosinophil counts were higher in females than males but the difference was not statistically significant ($p = 0.080$) and there was no significant interaction between sex and diet. The overall means were 44 ± 20 , 647 ± 391 , and $683 \pm 266 \times 10^3$ total eosinophils for the VAD, 4-IU, and 250-IU groups, respectively (Fig. 4). Both the 250-IU ($p = 0.013$) and the 4-IU ($p = 0.020$) group means were significantly greater than the VAD mean, but these means did not differ from one another ($p = 0.86$). Thus, the levels of pulmonary eosinophilia in the 4-IU and 250-IU diet groups were 14.7- and 15.5-fold greater than those in the VAD group, respectively.

In experiment 2, mean BAL eosinophil counts were again higher in the 4-IU and 250-IU diet groups than in the VAD group and were also higher in females than in males (Fig. 4). When sexes were analyzed together, the mean BAL eosinophil counts were 502 ± 109 , 974 ± 140 , and $1,231 \pm 143 \times 10^3$ in the VAD, 4-IU, and 250-IU groups, respectively. Thus, the 4-IU group mean was 1.9-fold greater than the VAD mean ($p = 0.13$) while the 250-IU mean was 2.5-fold greater ($p = 0.017$). BAL eosinophil counts were significantly greater ($p < 0.001$) in females than in males ($1,183 \pm 171$ vs $604 \pm 139 \times 10^3$). No significant interaction was seen between sex and diet. However, in female mice eosinophil counts were 2.6-fold greater in the 4-IU than in the VAD group ($p = 0.006$) and 3.0-fold greater in the 250-IU group than in the VAD group ($p < 0.001$). The diet effect was not significant in male mice ($p = 0.54$) and the corresponding fold differences in

eosinophil counts were 1.0 (4-IU vs VAD group) and 1.7 (250-IU vs VAD group) (Fig. 4).

Blood differential counts. Differential percentage counts of lymphocytes, monocytes, granulocytes, and eosinophils were made in three male and three female mice receiving aerosol OVA challenge from each diet group in the second experiment ($n = 18$ mice). No diet differences were seen for lymphocytes, monocytes, or neutrophils (data not shown). The percentage of eosinophilia was greater in females (1.07 ± 0.26) than in males (0.17 ± 0.26 ; $p = 0.035$) and the interaction between diet and sex was of marginal statistical significance ($p = 0.064$). In male mice the percentage of eosinophilia did not differ by diet ($p = 0.42$), with the means in the VAD, 4-IU, and 250-IU groups being 0 ± 0 , 0.51 ± 0.51 , and $0 \pm 0\%$, respectively. However, in female mice the means did differ by diet ($p = 0.046$), with the mean in the VAD group (2.57 ± 0.84) being significantly greater than the mean in either the 4-IU group (0.47 ± 0.47 ; $p = 0.039$) or the 250-IU group (0.17 ± 0.17 ; $p = 0.024$). Thus, in direct contrast to the finding in BAL, the percentage of whole-blood eosinophilia in the 4-IU and 250-IU groups were only 0.18-fold and 0.065-fold of the level seen in the VAD group.

BAL macrophages. Macrophage counts did not differ as a result of aerosol treatment (OVA vs sham) or dietary treatment in either experiment (Fig. 4). Macrophage counts were higher in females than males ($p < 0.001$) in the second experiment (0.110 ± 0.016 vs $0.058 \pm 0.014 \times 10^6$ cells).

Lung histopathology. Lung sections stained with H&E showed a significant eosinophilic response in OVA aerosol-challenged mice fed the 250-IU diet (Fig. 5C), but not in the OVA aerosol-challenged VAD mice (Fig. 5A). Also, compared with mice fed 4 IU of vitamin A (Fig. 5B), it appeared, qualitatively, that more eosinophils were located in the lung parenchyma near venules or airways, reflecting already the increase of eosinophils in the BAL fluid. As expected, eosinophilia did not develop in mice being not exposed to nebulized OVA (Fig. 5D).

VAD decreases BAL IL-4 and IL-5 concentrations

Cytokine concentrations were undetectable in most BAL specimens from mice receiving the AIR treatment (data not shown). Thus, all comparison presented here are from mice challenged with nebulized OVA.

In the first experiment BAL cytokine concentrations did not differ by sex and no interactions were seen between sex and diet. The BAL IL-4 concentration was 18.7-fold greater in the 4-IU group ($p = 0.025$) than in the VAD group and was 23.8-fold greater in the 250-IU ($p = 0.023$) group than in the VAD group. The 4-IU and 250-IU groups did not differ from one another ($p = 0.99$) (Fig. 6). Similarly, the IL-5 concentration was 12.4-fold greater in the 4-IU group ($p = 0.023$) than in the VAD group and 30.7-fold greater in the 250-IU group ($p = 0.003$) than in the VAD group. The 4-IU and 250-IU groups did not differ from one another ($p = 0.24$). TNF- α concentrations showed the opposite trend, tending to be higher in the VAD group, but differences among the diet groups were of marginal statistical significance ($p = 0.051$).

In the second experiment, BAL IL-4 concentrations differed significantly by diet ($p = 0.010$) and sex ($p = 0.002$, 112 ± 17 pg/ml in females vs 40 ± 13 pg/ml in males) (Fig. 6). There was no significant diet $\times$ sex interaction. The mean IL-4 concentration in the 4-IU group was 2.1-fold greater ($p = 0.23$) than in the VAD group, while in the 250-IU group it was 4.4-fold greater ($p = 0.0026$). The 4-IU and 250-IU groups did not differ from one another ($p = 0.14$). When females were analyzed separately, the mean IL-4 concentration in the 4-IU group was 3.1-fold greater ($p = 0.21$) than in the VAD

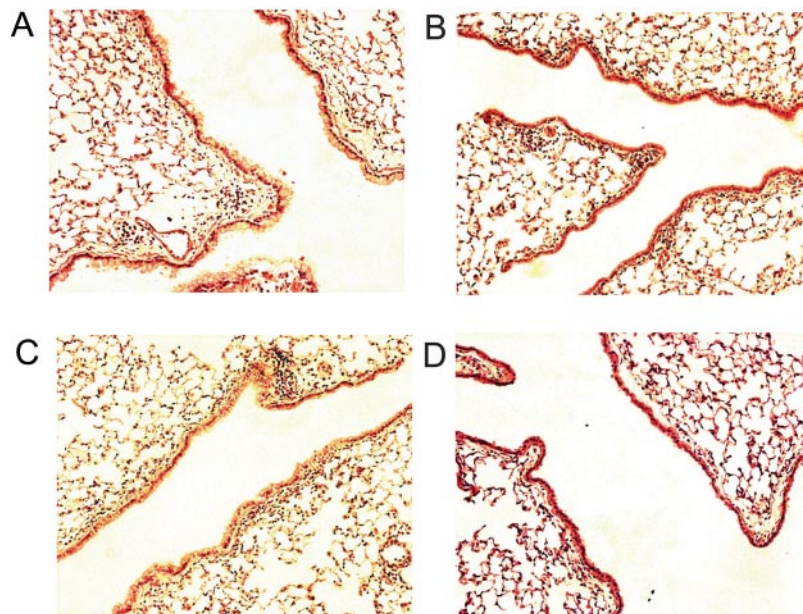


FIGURE 5. Vitamin A exacerbates OVA-induced eosinophilic lung inflammation. OVA-sensitized C57BL/6 mice were either challenged with nebulized OVA or sham treatment (AIR control). Paraffin sections (original magnification, $\times 40$) of secondary to tertiary airways that branch from the primary lobar bronchus were stained with H&E. A, OVA-challenged VAD mice; B, OVA-challenged mice fed the 4-IU diet; C, OVA-challenged mice fed 250-IU diet; D, AIR control mice fed the VAD diet.

group, while in the 250-IU group it was 4.9-fold greater ($p = 0.0047$). The 4-IU and 250-IU groups did not differ from one another ($p = 0.26$). When males were analyzed separately, IL-4 concentrations did not differ by diet ($p = 0.17$). Similarly, BAL IL-5 concentrations differed significantly by diet ($p = 0.031$) and sex ($p < 0.001$; 184 ± 22 pg/ml in females vs 20 ± 16 pg/ml in males) (Fig. 6). The diet $\times$ sex interaction was of marginal statistical significance ($p = 0.056$). The mean IL-5 concentration in the 4-IU group was 8.5-fold greater ($p = 0.012$) than in the VAD group, while in the 250-IU group it was 2.5-fold greater ($p = 0.075$). The 4-IU and 250-IU groups did not differ from one another ($p = 0.31$). When females were analyzed separately, the mean IL-4 concentration in the 4-IU group was 12.0-fold greater ($p = 0.002$) than in the VAD group, while in the 250-IU

group it was 27.6-fold greater ($p = 0.0019$). The 4-IU and 250-IU groups did not differ from one another ($p = 0.061$). When males were analyzed separately, IL-4 concentrations did not differ by diet ($p = 0.88$). TNF- α BAL concentrations were significantly lower ($p = 0.033$) for female than male mice (7.3 ± 4.6 vs 18.0 ± 3.4 pg/ml), but no difference was seen due to diet ($p = 0.46$). IP-10 was also measured in BAL samples and was higher in OVA-treated rather than AIR mice, but concentrations did not differ by sex or diet (data not shown).

Pulmonary function studies

Because airway hyperresponsiveness (AHR) is a fundamental feature of asthma, we compared the total lung resistance and dynamic compliance at baseline and after the inhalation of methacholine in mice on the different vitamin A diets. Statistical comparisons were made to determine the effect of vitamin A status on resistance and compliance at baseline and after the inhalation of saline (vehicle) and serial low doses of methacholine (0.5, 1.0, and 2.0 mg/ml). Comparisons were made separately for experiments 1 and 2. Data are shown in Fig. 7 and a summary of statistical findings is presented in Table I. Sex of the mice did not affect any of these measures of pulmonary function, and no sex $\times$ diet interactions were seen.

Effect of vitamin A status on resistance and compliance in AIR control groups

In the first experiment, diet did not affect any pulmonary function endpoint in the AIR control group. However, in the second experiment, although no differences were seen for resistance, compliance values differed significantly among all diet groups across the entire dose-response curve (Fig. 7 and Table I). Compliance was highest for the 4-IU control diet and lowest for the VAD diet.

Vitamin A deficiency blocks OVA-induced changes in airway responsiveness

OVA immunization and aerosol treatment normally induces increased AHR and decreased compliance in response to methacholine challenge. Such changes are one of the hallmarks of the OVA model of asthma. In contrast to the 4-IU and 250-IU diet groups, no significant increase in resistance or decrease in compliance was seen at any methacholine dose in either experiment for the VAD

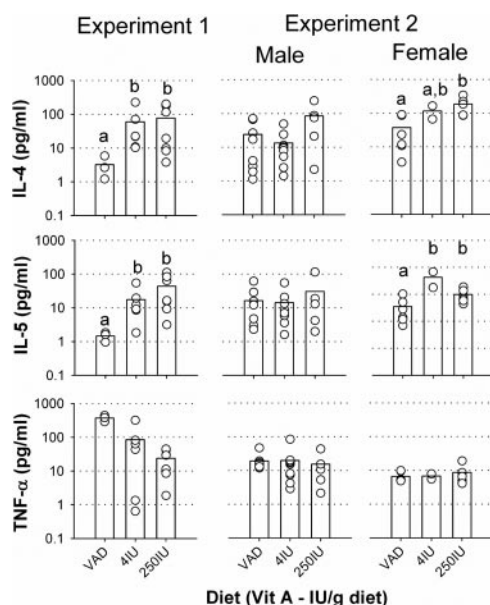


FIGURE 6. Bronchoalveolar lavage cytokine concentrations in mice fed different levels of vitamin A (Vit A) (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet). Within each figure different superscript letters indicate that group means are significantly different. Bars indicate means whereas data points represent results from individual mice.

Experiment 1

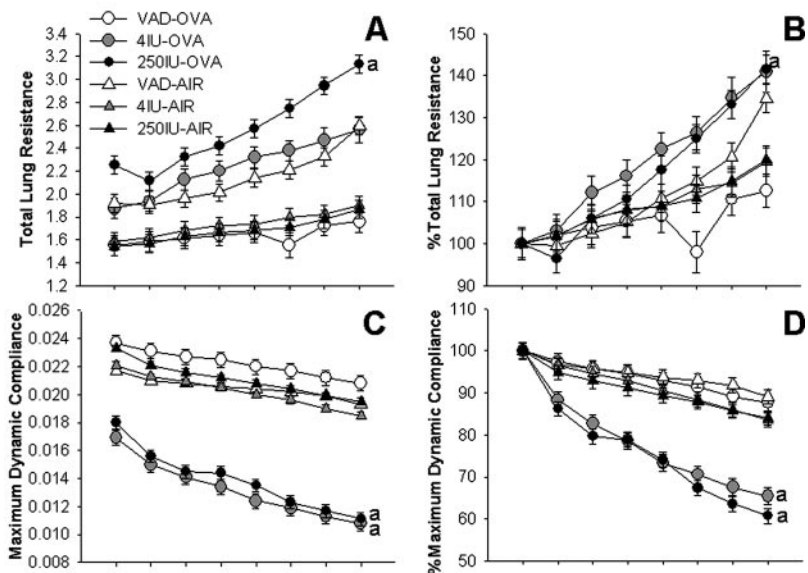


FIGURE 7. Airway responsiveness in mice fed different levels of vitamin A (VAD: 0 IU/g diet in experiment 1 and 0.2 IU/g in experiment 2; 4-IU/g diet and 250-IU/g diet) and challenged with OVA aerosol or a sham aerosol treatment, AIR. Baseline resistance was measured before administering saline and serial doses of methacholine (0.5, 1.0, and 2.0 mg/L). The graph shows mean values $\pm$ SEM for two male and two female mice (except for one male and two females for VAD-OVA in experiment 1) taken over 3 min during and following (Post) methacholine administration in experiment 1 and experiment 2. In experiment 2, the development of respiratory distress in three of four mice in the 250 IU-OVA group prompted termination of data collection at the two highest MCh doses, and data from these doses were not used in statistical analysis. A and E, resistance; B and F, resistance as the percentage of increase from the baseline value (100%); C and G, compliance; D and H, compliance as the percentage of decrease from the baseline value in the first and second experiment, respectively. Lower case letters indicate differences vs VAD-OVA group (a) or vs 4-IU group (b). Other statistical comparisons are discussed in Results.

mice (Fig. 7 and Table I). In addition, in the second experiment lung resistance was significantly greater among VAD mice in the AIR control group as compared with the OVA group, the opposite of what was expected.

Effect of vitamin A status on resistance and compliance in OVA treatment groups

Total lung resistance was significantly greater in the 250-IU group than in the VAD group while compliance was significantly lower in the 250-IU diet group compared with the VAD group (Fig. 7 and Table I). The changes in lung function in the OVA-exposed animals were significantly worse in the 250-IU group than in the VAD group. Only minor variability was seen between experiments. Similarly, significant differences in both resistance and compliance measures were seen between the 4-IU and VAD groups, although minor differences were seen between the first and second experiments. For example, the greater resistance and per-

centage of resistance seen in the 4-IU vs VAD group were statistically significant only in experiment 2, while the lower compliance and percent compliance of the 4-IU group were statistically significant only in experiment 1 (Table I).

The most significant changes in lung function were seen in the mice fed the 250-IU diet, particularly in the second experiment; e.g., the percentage change in compliance after MCh challenge was significantly lower in the 250-IU group compared with the 4-IU control group. (Fig. 7 and Table I). Also in experiment 2, three of the four mice in the 250-IU OVA group developed respiratory distress and could not be adequately ventilated at the penultimate methacholine dose (1.0 mg/ml) and data collection was terminated. This did not occur in other diet groups in either experiment.

Discussion

In this study, we show for the first time that VAD, produced by a strict diet deficient in vitamin A, prevents the development of

Table I. Comparisons of airway resistance (R), percentage of resistance (% R), compliance (C), and percentage of compliance (% C) among three groups of mice: VAD, control (4 IU/g), and high-level vitamin A (250 IU/g)^a

Diet Comparisons within Air Treatment								
Comparison	Experiment 1				Experiment 2			
	R	% R	C	% C	R	% R	C	% C
250 IU vs VAD	NS	NS	NS	NS	NS	NS	All	NS
4 IU vs VAD	NS	NS	NS	NS	NS	NS	All	NS
250 IU vs 4 IU	NS	NS	NS	NS	NS	NS	All	NS

Diet Comparisons with OVA Treatment								
Comparison	Experiment 1				Experiment 2			
	R	% R	C	% C	R	% R	C	% C
250 IU vs VAD ^b	1-2-2p	2p	All	All	0.5p-1p	1-1p	1-1p	0.5-1p
4 IU vs VAD	NS	NS	All	All	2-2p	2-2p	NS	NS
250 IU vs 4 IU ^b	NS	NS	NS	NS	NS	NS	NS	All

Air vs OVA Comparisons within Diets								
Comparison	Experiment 1				Experiment 2			
	R	% R	C	% C	R	% R	C	% C
250 IU ^b	All	NS	All	All	0.5p-1p	0.5p-1p	All	All
4 IU	1-2p	NS	All	All	NS	NS	All	2p
VAD	2p	2p	NS	NS	NS	NS	NS	NS

^a Mice were immunized with OVA, sensitized by aerosol with OVA or sham treatment (AIR), and challenged with graded levels of Mch (baseline (b), saline (s), 0.5 mg/ml, 0.5-p, 1.0, 1.0p, 2.0, 2.0p; where "p" indicates post-Mch administration). Significant differences ($p < 0.05$; NS, not significant) for the indicated comparisons were identified by two-way repeated measures ANOVA at each challenge level. Differences at individual challenge levels are indicated by the following notations: "b, s, 0.5, 0.5p, 1, 1p, 2, 2p." If all challenge levels differ significantly, this is indicated by "All".

^b For experiment 2, comparisons were made only at doses b, s, 0.5, 0.5p, 1, and 1p because three of four mice in the 250-IU diet group developed respiratory distress at 2.0 mg/mL methacholine and data collection was thus terminated at this dose.

OVA-induced allergic airway inflammation and hyperresponsiveness that are hallmarks of asthma. VAD also reduced other markers of pulmonary inflammation relative to mice fed the 4-IU control diet and the high-level 250-IU diet, including BAL eosinophil numbers and the levels of the Th2 cytokines IL-4 and IL-5 in BAL samples. VAD also reduced serum IgE and IgG1 responses when compared with these two diets. Although this result suggests that VAD may decrease the risk or severity of asthma in humans, it should be remembered that VAD impairs protective responses, including Th2-mediated gut helminth expulsion (4, 5) in an animal model. VAD is also associated with increased mortality from infectious diseases in infants and young children in developing countries (2). Thus, while reduction of airway inflammation by VAD may decrease the severity of experimental asthma, such a deficit could well be harmful when Th2 responses are protective rather than pathologic.

We also observed that the high-level vitamin A diet produced a greater IgE response and more significant changes in AHR after OVA exposure compared with the 4-IU diet, particularly in the second experiment. Thus, it appears that the development of allergic inflammation and AHR is enhanced by the contribution of high-level dietary vitamin A. This diet clearly represents excessive intake and raises the legitimate question of how excessive intake might affect the incidence or severity of asthma in humans. Early childhood use of multivitamin supplements (12) as well as supplements containing only vitamins A and D (13) have both been associated with an increased risk of asthma later in life. Similarly, high intake and high body stores of vitamin A might increase the severity of asthma in children or adults. This possibility warrants further investigation, but the present observation certainly cautions

against the overuse of vitamin A supplements, particularly by those predisposed to atopic disease. In brief, more may not always be better with regard to vitamin A intake.

Because high-level dietary vitamin A increases eosinophilic inflammation in this mouse model of asthma, it is interesting to note that use of the vitamin A metabolite retinoic acid to treat acute promyelocytic leukemia results in respiratory distress and pulmonary neutrophilic inflammation in ~10–15% of patients (21). It is possible that this syndrome (termed "retinoic acid syndrome") and the enhanced eosinophilic inflammation may have similar underlying mechanisms, perhaps including enhanced granulocyte development and trafficking from the bone marrow to the lung. Because only a subset of patients develop this syndrome, this suggests that host factors might also predispose healthy subjects to adverse consequences of high vitamin A intake.

Mice were fed diets with different levels of vitamin A. Male mice showed severe VAD in the first experiment as indicated by a significant drop in body weight relative to the 4-IU control group. Therefore, in the second experiment, mice in the VAD group received a minimal amount of dietary vitamin A (0.2 IU/g). This amount was sufficient to achieve depleted stores of vitamin A in the liver without affecting their body weights. Vitamin A status differed among all the diet groups in both experiments as indicated by the differences in liver levels and the principal body storage site (19, 22), as well as lung levels. The liver storage we observed in mice kept on a 4-IU diet was comparable to other studies with mice fed regular chow (23, 24). We did not observe a significant weight change in mice fed a 250-IU diet relative to the 4-IU control group. The high pulmonary levels of vitamin A in the 250-IU

group could directly exacerbate airway inflammation and hyperresponsiveness, perhaps by increasing the local availability of retinoic acid as has been suggested previously (25, 26).

In this study, we show that VAD impairs Th2-driven serum IgE and IgG1 responses and that the high-level diet can enhance at least the IgE response. These findings are in agreement with previous results showing that supplementation with vitamin A in a murine sensitization model with topical application of dinitrochlorobenzene enhances inflammatory responses that are accompanied by decreased Th1 responses (27) and with the general notion that supplementation with vitamin A depresses IFN- γ and favors Th2 responses (5, 7, 28).

The recruitment of eosinophils to the airways is perhaps the most characteristic feature of airway inflammation in chronic allergic asthmatics (29). This study shows that compared with mice on control and high-level vitamin A diets, VAD mice had significantly fewer BAL eosinophils. Eosinophilia is driven by a Th2 response and involves local production of IL-4 and IL-5 (30). It was thus not surprising that BAL IL-4 and IL-5 levels also differed by dietary treatment in parallel with changes in eosinophil counts.

Mature eosinophils are specifically mobilized from the bone marrow by the combined actions of IL-5 and eotaxin (31, 32). They are primed in the circulation, increasing their migratory capacity and adhesiveness. Recruitment of eosinophils within the lung contributes to both AHR and airway remodeling (33). VAD female mice had a significantly higher percentage of circulating eosinophils in the bloodstream, but fewer eosinophils in the BAL. This suggests that VAD might impair the recruitment or migration of eosinophils into the lungs. This might be due to the inhibited expression of cell surface integrins as well as their ligands. Eosinophil recruitment into the lung interstitium and then to the airway lumen is generally dependent on α_4 , β_1 , and β_2 integrins (34–37). $\alpha_4\beta_7$ integrins are found in circulating eosinophils and in the gastrointestinal tract, whereas lung eosinophils lose their surface expression of β_7 integrin when passing through the lung tissue (36). Retinoic acid can induce $\alpha_4\beta_7$ integrin expression on lymphocytes, and VAD in mice causes a reduction in T lymphocytes in the gut lamina propria (but not lung) and a decrease in the numbers of $\alpha_4\beta_7^+$ lymphocytes (38, 39). It is currently not known whether retinoic acid treatment or VAD can influence the distribution of other integrins, such as $\alpha_4\beta_1$. Additional studies are necessary to determine whether VAD causes impaired recruitment of eosinophils to the lung due to the lack of integrins, which could also lead to the higher percentage of circulating eosinophils seen here. In addition, the expression of eotaxins by the airway epithelium could be inhibited by VAD (29).

VAD inhibited the development of OVA-induced pulmonary hyperresponsiveness in these experiments. VAD mice exposed to OVA aerosols did not show greater responsiveness to MCh challenge than the VAD-AIR mice. However, OVA aerosol treatment, as expected, did induce such hyperresponsiveness in mice fed both the 4-IU control diet and the 250-IU high-level vitamin A diet. Similarly, VAD-OVA mice had lower BAL eosinophil counts and lower BAL Th2 cytokine levels than did the 4 IU-OVA and 250 IU-OVA mice, suggesting that VAD decreased pulmonary hyperresponsiveness by decreasing pulmonary eosinophilic inflammation. Unexpectedly, the VAD-AIR mice in experiment 1 appeared to have greater pulmonary resistance in response to MCh challenge than did the VAD-OVA mice, exactly the opposite of the usual situation in mice on adequate vitamin A diets. However, this difference was statistically significant only at the highest level of the dose-response curve (Table I) and was not seen again in experiment 2, suggesting that the finding was spurious.

In contrast to the present findings, other work has shown that VAD, independent of pulmonary inflammation, increases airway hyperresponsiveness to MCh challenge in rats by diminishing pulmonary expression of inhibitory muscarinic receptor-2 and elastic fibers (40–43). These data suggest that the VAD-AIR mice in the present study might have increased airway resistance relative to the 4 IU-AIR or 250 IU-AIR mice, but such differences were not seen, although lower compliance was seen in experiment 2 and may be related to the same mechanism.

We acknowledged that alterations in breathing frequency and tidal volume during our protocol could affect our true compliance and resistance measurements because they are based on an ideal single compartment model (44, 45). Specifically, the contribution of subsegmental atelectasis that occurs with the nebulization of Mch cannot be discounted and this may affect our true resistance and compliance measurements. However, the relative differences between groups should not be affected by any atelectasis that might develop during the duration of the protocol, and we believe our comparisons are valid.

In the second experiment, more eosinophils and macrophages were recruited to the lungs in the mice exposed to OVA, and this was associated with a significant increase in IL-4 and IL-5 levels but a decrease in TNF- α in the BAL of female mice. Previous studies have shown that female mice develop a greater lung inflammatory response upon exposure to OVA compared with male mice, but the differences are modest (46). Our results suggest that vitamin A may contribute to the sex difference in this model. Recent evidence indicates that estrogens inhibit the production of Th1 proinflammatory cytokines, whereas they stimulate the production of Th2 anti-inflammatory cytokines. This can explain why estrogen suppresses and potentiates Th2-mediated diseases such as asthma and allergic rhinitis (reviewed in Ref. 47). This is in line with our data suggesting that female mice seem to have a shift toward a Th2 phenotype.

It is noteworthy that many epidemiological studies suggest that the prevalence of asthma, particularly severe asthma, is higher in adult women than in adult men. These gender differences in humans appear to be the product of sociocultural and environmental differences as well as biological differences that include genetic, pulmonary, hormonal, and immunological factors (48, 49).

In summary, our data demonstrate that VAD decreased OVA-induced synthesis of plasma IgE and DNP-specific IgG1, recruitment of eosinophils, and IL-4 and IL-5 release into the BAL fluid as well as lung hyperresponsiveness. Mice fed 250 IU/g vitamin A showed severe lung hyperreactivity and inflammation that was associated with increased airway eosinophilia and Th2 cytokines. This study showed that high dietary levels of vitamin A augmented the severity of experimental asthma and modulated Th1/Th2 development with a shift toward Th2, which may account for the increased asthma severity.

Acknowledgments

We thank Xiaowen Jiang, Lisa Franzi, Erin M. O'Roark, and Alina Wettstein for expert technical assistance and Paula Do for assistance in manuscript preparation.

Disclosures

The authors have no financial conflict of interest.

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