

Dietary Cholesterol Enhances Pulmonary Eosinophilic Inflammation in a Murine Model of Asthma

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Key Words

Asthma · Cholesterol · Interleukin-5 · Diet · Inflammation

Abstract

Background: Epidemiological studies have suggested that the dietary pattern may be associated with the prevalence of asthma. We previously reported that an increased intake of foods of animal origin was associated with the occurrence of allergic rhinitis and asthma in adolescents. Here we examined the effect of dietary cholesterol in a murine model of allergic pulmonary inflammation. **Methods:** Weanling C57BL/6 mice were fed a control diet containing 0.02% cholesterol or a diet supplemented with 1% or 2% cholesterol. Four weeks later the mice were sensitized with intraperitoneal ovalbumin (OVA) followed by OVA or saline inhalation 2 weeks later. OVA aerosol-induced inflammation was significantly enhanced by dietary supplementation of 1% or 2% cholesterol. **Results:** Among OVA-challenged mice, leukocyte numbers, particularly those of eosinophils, in the bronchoalveolar space increased by 3- to 5-fold with the cholesterol supplement. Among OVA aerosol-challenged mice, the levels of interleukin-5 and cysteinyl leukotrienes in the bronchoalveolar lavage fluid were significantly higher in those fed the 2% cholesterol diet com-

pared with mice on the control diet. **Conclusions:** Dietary cholesterol may enhance pulmonary allergic inflammation.

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Introduction

The prevalence of childhood asthma has increased in many developed and developing countries in the past decades. This phenomenon is most likely the result of complex interactions between multiple environmental and genetic factors [1, 2]. Several hypotheses have been raised, among them the changes in the dietary pattern, particularly the increased consumption of unsaturated fatty acids [3, 4]. In a cross-sectional survey in Taiwan, the prevalence of allergic rhinitis and asthma in adolescents was associated with the intake frequency of several food items, including liver, deep-fried foods and meat [5]. The historical trend of food intake also suggested an association between dietary fats and asthma prevalence. Dietary fat composition in Taiwan increased from 16% of the daily calories in 1950 to 36% in 1987 [6]. There was also a steady increase in the consumption of animal fats, including cholesterol, in the period from 1950 to 1990 [7]. Cholesterol is an important composition of mammalian

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1018–2438/01/1254–0329\$17.50/0

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cell membranes, and may play a critical physiological role by modulating the fluidity of the membrane or by direct interaction with membrane proteins [8]. Whether dietary cholesterol affects allergic inflammation has not been explored. Here we used a murine model of allergic asthma to test the hypothesis that dietary cholesterol may have an effect on pulmonary inflammatory processes. We examined the severity of inflammation and the expression of several inflammatory mediators in mice fed diets containing various percentages of cholesterol.

Materials and Methods

Animal Sensitization and Challenge

Male C57BL/6 mice were obtained from in-house breeding at the animal facility of the Yang-Ming University at 4 weeks of age. The animal facility was maintained at 22–24 °C with a 12-hour dark/light cycle. In the first experiment, weanling C57BL/6 mice (5th week) were fed either a control diet containing 200 PPM cholesterol (Rodent Diet 5001, Lab Diet, Brentwood, Mo.), or a diet supplemented with 1% (wt/wt) cholesterol (Sigma, St. Louis, Mo.). The mice were sensitized at the 9th week by intraperitoneal injection of 50 µg ovalbumin (OVA; grade V, Sigma) with 4 mg alum, and boosted 7 days later with the same dose of OVA. Fourteen days after sensitization, the mice were exposed to aerosols of 2% OVA in saline or saline only, 10 min per day for 3 consecutive days. The aerosols were generated by a DeVilbiss nebulizer driven by an air compressor (PulmoAide, DeVilbiss, Somerset, Pa.). Blood collection and bronchoalveolar lavage (BAL) were done 48 h after the last aerosol inhalation. After BAL, the lungs were fixed in formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. In the second experiment, a similar protocol was adopted except that the mice were not boosted 7 days after sensitization, and they were fed either a control diet or a diet supplemented with 2% (wt/wt) cholesterol.

Bronchoalveolar Lavage

Forty-eight hours after the last aerosol inhalation, peripheral blood was collected from the tails of the animals and the sera were kept at –20 °C until analysis. The animals were then euthanized with sodium pentobarbital. BAL was performed with 5 washes of 0.6 ml phosphate-buffered saline (PBS). The first lavage was centrifuged at 300 g for 10 min, and the supernatant was kept at –70 °C. Cells in the first wash were combined with the remaining washes and counted. Cytospin slides were fixed and stained with the Hemacolor Stain Set (EM Science, Gibbstown, N.J.), and at least 200 cells were counted for leukocyte differential counts. After lavage, the chest was opened, and the lungs were removed and kept in buffered formalin. Tissue sections were stained with hematoxylin and eosin. The identity of eosinophils in some slides was confirmed by fast green staining (eosinophil granules stained with 0.2% fast green in 70% ethanol for 3 min, nuclei counterstained with 0.5% neutral red in distilled water for 2 min).

Serum Cholesterol

Total serum cholesterol concentration was measured by colorimetric procedures. The reagents were provided by cholesterol E-test

kit (Wako Pure Chemical Industries, Osaka, Japan). Briefly, free cholesterol in serum and cholesterol released from cholesterol esterase hydrolysis were oxidized with cholesterol oxidase to produce H₂O₂, which in the presence of oxidase, 4-aminoantipyrine, and the substrate 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfoethyl)-aminotoluene formed a blue product that was quantified by spectrophotometry at 600 nm.

ELISA for OVA-Specific IgE

Ninety-six-well EIA plates (Costar, Corning, N.Y.) were coated with 2 µg/ml rat anti-mouse IgE (Zymed, San Francisco, Calif.) in 0.1 M carbonate buffer (pH 9.5) at 4 °C overnight. The plates were blocked with 1% BSA in PBS-0.5% Tween 20 for 1 h, washed with PBS-0.05% Tween 20, and incubated with mice serum (1:10) for 2 h at room temperature. Captured OVA-specific IgE was detected by 1 µg/ml biotinylated OVA (1 h at room temperature), followed by 1:1,000 streptavidin-conjugated peroxidase (Sigma). Sera from OVA-sensitized and OVA-challenged mice were pooled, and serial 1:2 dilutions of the pooled sera used for a standard curve. The IgE value of each serum sample was expressed as the percentage of the pooled sera.

Interleukin-5, Tumor Necrosis Factor-α and Leukotrienes

Serum and BAL fluid (BALF) interleukin-5 (IL-5), and BALF tumor necrosis factor-α (TNF-α) were measured by the ELISA kit, according to the instruction of the manufacturer (Endogen, Woburn, Mass.). Cysteinyl leukotrienes (leukotrienes C₄, D₄, E₄, C₅, D₅ and E₅) in BALF were measured by the Cysteinyl-Leukotriene Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, Mich.). The detection limit was 5 pg/ml for IL-5, 10 pg/ml for TNF-α and 3.24 pg/ml for cysteinyl leukotrienes.

Statistical Analysis

Normality was tested for data distribution. If data of a variable fit normal distribution, the data were expressed as mean ± SD, and analysis of variance was applied to compare the difference between groups. For variables that were not normally distributed (such as IL-5 levels in serum and BALF, leukotriene levels in BALF), the Wilcoxon rank sum test was applied to compare differences between groups. A difference with p values less than 0.05 was considered statistically significant. All statistical analyses were made using the Statistical Analysis System package (SAS 6.12, SAS Institute, Cary, N.C.).

Results

Effect of Cholesterol on Serum Cholesterol Level and Body Weight

According to our protocol, the mice were fed different diets for 6½ weeks at the end of each experiment, when blood was collected. Adding cholesterol to the diet did not significantly affect the body weight. Serum levels of cholesterol were 34 and 57% higher in mice fed a diet supplemented with 1% or 2% cholesterol, respectively (p < 0.01, table 1). Compared with saline inhalation, OVA aerosol challenge did not affect serum cholesterol.

Table 1. Serum cholesterol level (mg/dl) and body weight (g) of mice in two different runs of the experiment with the 1% or 2% cholesterol diet and control diet

Diet	Inhalation	n	Serum cholesterol ¹	Body weight
Control	Saline	7	83.1 ± 17.0	18.7 ± 1.4
	OVA	9	91.5 ± 9.9	18.7 ± 1.8
1% cholesterol	Saline	7	134.6 ± 62.4	19.4 ± 1.4
	OVA	9	106.9 ± 21.2	19.7 ± 1.4
Control	Saline	5	108.84 ± 17.54	20.0 ± 1.1
	OVA	6	130.29 ± 18.06	21.2 ± 1.7
2% cholesterol	Saline	6	172.44 ± 24.32	20.2 ± 1.9
	OVA	5	162.55 ± 24.46	19.7 ± 0.6

¹ p < 0.05 1% and 2% cholesterol groups vs. their respective control diet groups.

Table 2. Leukocyte numbers per mouse (× 10⁴ cells, mean ± SD) and differential count (mean % in parentheses) in BALF of mice in two separate runs of the experiment comparing the 1% or 2% cholesterol diet and control diet

Diet	Inhalation	Total cell	Macrophage	Eosinophil	Neutrophil	Lymphocyte
Control	Saline (n = 7)	25.4 ± 14.9	23.3 ± 13.7 (91.7)	0.6 ± 0.5 (3.6)	0.0 ± 0.0 (0.1)	1.5 ± 1.4 (4.6)
	OVA ¹ (n = 9)	134 ± 57	43.6 ± 12.6 (33.4)	72.7 ± 41.6 (55.3)	0.0 ± 0.0 (0.1)	14.4 ± 9.7 (9.5)
1% cholesterol	Saline (n = 7)	22.8 ± 12.2	21.8 ± 12.6 (93.5)	0.2 ± 0.2 (1.6)	0.1 ± 0.1 (0.3)	0.8 ± 0.7 (4.6)
	OVA ² (n = 9)	718 ± 483	62.3 ± 33.6 (11.9)	581 ± 414 (77.8)	7.3 ± 7.7 (1.1)	68.2 ± 54.4 (9.1)
Control	Saline (n = 5)	44.7 ± 14.3	43.3 ± 14.1 (96.9)	0.0 ± 0.0 (0)	1.0 ± 0.0 (0)	1.3 ± 1.0 (3.1)
	OVA ¹ (n = 6)	82.3 ± 31.9	20.0 ± 8.8 (29.7)	41.4 ± 27.6 (45.8)	5.9 ± 5.2 (6.7)	14.9 ± 7.1 (17.8)
2% cholesterol	Saline (n = 6)	50.4 ± 8.5	49.5 ± 8.7 (98.1)	0.0 ± 0.0 (0)	0.1 ± 0.1 (0.17)	0.8 ± 0.7 (1.8)
	OVA ² (n = 5)	242 ± 104	27.7 ± 9.6 (12.5)	161 ± 78 (65.5)	28.8 ± 18.5 (11.1)	24.4 ± 5.8 (10.9)

¹ Total cell, eosinophil and lymphocyte numbers significantly higher than in the saline group with the control diet (p < 0.01).

² Total cell, eosinophil, neutrophil and lymphocyte numbers of OVA-challenge groups with 1% or 2% cholesterol supplement significantly higher than in the OVA group with the control diet (p < 0.01).

Cholesterol Effect on Pulmonary Inflammation

All mice were sensitized with intraperitoneal OVA, and exposed to OVA or saline aerosol 2 weeks later. OVA inhalation caused a significant increase of eosinophils and lymphocytes in the BALF, demonstrating the expected effect of antigen challenge. The histology of OVA-challenged mice showed prominent alveolar, peribronchial and perivascular eosinophil and mononuclear infiltration in both dietary groups. Dietary cholesterol did not cause discernable changes in airway morphology in saline-challenged mice. Table 2 shows the results from separate runs of experiments with 1% or 2% cholesterol. Mice fed a cholesterol diet had exaggerated responses to OVA inhalation. The numbers of eosinophils, neutrophils and lymphocytes were all significantly higher than in OVA-challenged mice fed a control diet. Compared with their respective control diet groups, the eosinophil numbers in

the BALF were 8- and 4-fold higher in mice fed 1% and 2% cholesterol. There was a minor difference in the immunization protocol, i.e. the booster OVA injection 1 week after sensitization was only performed in the experiment using 1% cholesterol, therefore the effect of 1% and 2% cholesterol could not be directly compared.

Dietary Cholesterol Increased IL-5 in BALF

Several inflammatory mediators in serum and/or BALF were measured in the experiment comparing control and 2% cholesterol diets. In mice not challenged with OVA inhalation, serum IL-5 was below the detection limit (5 pg/ml). OVA aerosol challenge caused elevation of serum IL-5 levels (p < 0.05) in both dietary groups; there was no significant effect of 2% cholesterol. IL-5 in BALF was undetectable in most saline inhalation mice. In OVA-challenged mice, IL-5 in BALF was significantly elevated

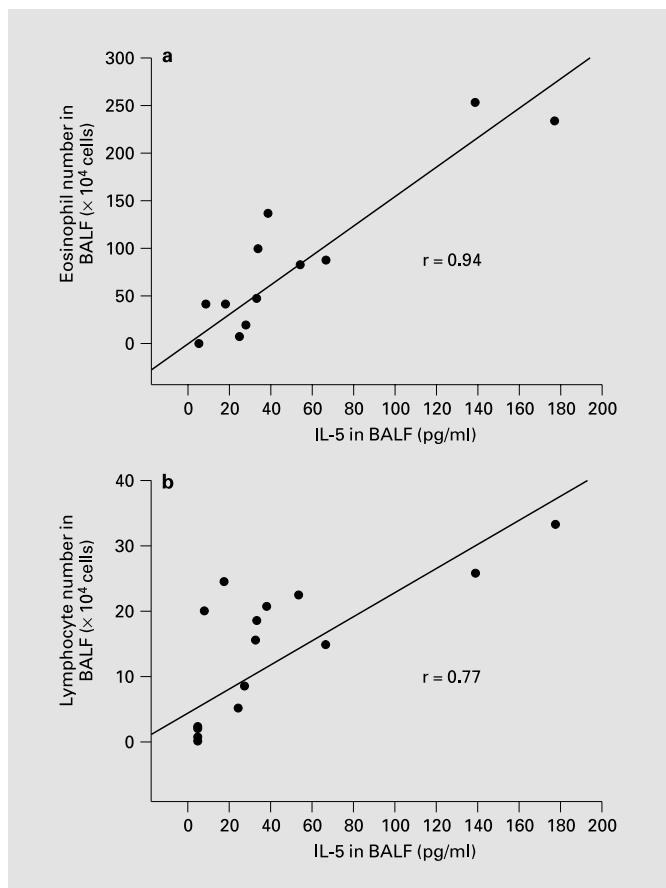


Fig. 3. Correlation between BALF IL-5 levels with eosinophils (a) and lymphocytes (b).

arate runs of experiments, that eosinophil infiltration was greatly increased by dietary cholesterol. Along with the increased number of eosinophils in lavage fluid, we also found increased IL-5 and cysteinyl leukotrienes in the bronchoalveolar compartment. With our experiment protocol, 2% dietary cholesterol raised the serum cholesterol level by 57%, but the mice were not overtly hyperlipidemic.

The recruitment of eosinophils into the alveolar space involves many steps, including antigen presentation, lymphocyte activation, chemokine release, leukocyte-endothelial interaction and leukocyte migration. Among the various inflammatory mediators involved, IL-5 has been implicated as the pivotal cytokine in eosinophil production in bone marrow and recruitment to the lungs. In this study, IL-5 production was enhanced in mice fed a diet with 2% cholesterol, and IL-5 concentrations in BALF correlated well with the numbers of eosinophils and lym-

phocytes. As both T lymphocytes and eosinophils produce IL-5, further studies are necessary to elucidate the role of this cytokine in the cholesterol-enhanced inflammatory reaction. Whether increased IL-5 production indicated T cell activation or merely reflected the higher number of inflammatory cells in the airspace is currently unknown.

How cholesterol affected eosinophil recruitment awaits further investigation. Cholesterol is a prominent component of mammalian cell membranes and may have a regulatory role in many membrane functions, either by modulation of membrane fluidity or by specific interaction with membrane proteins [8]. The membrane composition of lymphocytes from subjects with hypercholesterolemia was altered; phospholipids and polyunsaturated fatty acids were decreased along with an increase in cholesterol [9]. The cholesterol level of the plasma membrane may regulate lateral organization and presentation efficiency of human leukocyte antigen (HLA) molecules in lymphoblastoid cells [10]. In one in vitro study, preincubation of human peripheral blood monocytes with cholesterol led to an increased surface expression of class II HLA molecules and enhanced antigen presentation functions [11]. Thus, antigen presentation and lymphocyte activation could theoretically be affected by serum cholesterol.

If dietary cholesterol enhanced antigen presentation or upregulated the production of Th2 cytokines, it follows that serum IgE concentration would increase with cholesterol supplement. We did not detect an elevation of serum OVA-specific IgE levels by cholesterol feeding. This may suggest that cholesterol enhances inflammation processes by antigen-nonspecific mechanisms, such as increasing leukocyte-endothelial adhesion or chemotaxis [12, 13]. Alternatively, with considerable variability in IgE levels within each treatment group, our experiment may not be sensitive enough to detect an existing effect.

In addition to lymphokines, other inflammatory mediators may also be modulated by cholesterol. Cysteinyl leukotrienes are potent bronchial constrictors and may also stimulate mucus secretion in the airways. In this study these lipid mediators were higher in OVA-challenged mice fed a 2% cholesterol diet. In human studies it was shown that, compared with healthy subjects, granulocytes from hypercholesterolemic patients had a significantly increased synthesis of LTB₄, LTC₄, LTD₄, and LTE₄ upon stimulation in vitro, and the exaggerated response was associated with altered calcium pump activity [14]. It is unknown whether the increased leukotriene production observed here in the cholesterol group reflected an effect that was based on changes in membrane

physiology, or an effect secondary to the presence of stronger cytokine stimulation.

The study was performed to establish the biological basis of the association between diet and allergic diseases in an epidemiological survey [5]. The results seemed to correlate with the epidemiological findings. However, the response in mice to dietary cholesterol may be different than in humans. In addition to subtle interspecies differences in immunological responses, the contrast between high- and low-cholesterol diet was exaggerated in the mouse model. The average cholesterol intake (300–400 mg/day) corresponded with about 0.1% of dry weight for a 2,000-calorie diet. Thus, 1% cholesterol in a mouse diet is roughly 10 times the level of daily intake for humans. Further studies are necessary to examine whether the proinflammatory effect can be detected for lower concentrations of cholesterol in the mouse. More importantly, does dietary cholesterol have proinflammatory properties in humans? A tentative answer to this question can be found in a study with children who were hypercholesterolemic, where a low-fat, low-cholesterol diet reduced lymphocyte

counts of the CD3, CD4 and CD8 subsets, which were originally higher than in normal controls [15]. However, more data is necessary before any diet intervention study in asthmatic patients is carried out.

In conclusion, we have demonstrated that supplementing cholesterol in the diet aggravated antigen-induced pulmonary inflammation in this murine model of asthma. As far as the authors know, this is the first report on the effect of dietary cholesterol on allergic inflammation. Given that cholesterol is a ubiquitous food component and is consumed in larger amounts nowadays than before, its potential role in aggravating the symptoms of allergic diseases deserves further evaluation.

Acknowledgment

The authors wish to thank Dr. Ho-Jen Peng at the Taipei Veterans General Hospital for helpful discussions, Dr. Li-Shun Shih at the Taipei Jen-Ai Hospital for pathology consultations, and Ms. Wen-Ling Cheng for technical support. This study was funded by the National Science Council, Taiwan (NSC-89-2320-B-010-045).

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